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INVESTIGATION OF SOME TECHNICAL ASPECTS OF HORSERADISH PEROXIDASE AND ALKALINE PHOSPHATASE CONJUGATES FOR USE IN ELISA TESTS

BY

© ROBIN KIM KING

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Investigation of Some Technical Aspects of Horseradish Peroxidase and Alkaline Phosphatase Conjugates for Use in ELISA Tests submitted by Robin Kim King in partial fulfillment of the requirements for the degree of Master of Science in Medical Microbiology.

Odona Morgante
Supervisor

David L J Tyrrell

Ronald Whitehouse

DATED June 1, 1982

DEDICATION

This thesis is dedicated to my mother and father who instilled the value of knowledge and achievement within me and to Brian, my husband, who gave me support and encouragement throughout the Master's program.

ABSTRACT

The following study was undertaken to produce enzyme-conjugates to be used for antigen and antibody detection of Coxsackieviruses B1-B6 and for the detection of Western Equine Encephalomyelitis virus in animal and arthropod tissues. Anti-Coxsackieviruses B1-B6 immune sera were obtained from guinea pigs immunized with viral antigens prepared in HeLa cells and anti-Western Equine Encephalomyelitis virus was prepared in mice using antigen prepared in suckling mouse brains. Anti-guinea pig and anti-mouse enzyme-labelled conjugates were prepared using horseradish peroxidase and alkaline phosphatase by a periodate and a one-step glutaraldehyde conjugation procedure respectively. The horseradish peroxidase conjugate prepared failed to react in the ELISA test and yields were very low with little enzymatic activity. Conjugates prepared using alkaline phosphatase had enzymatic activities ranging from 195-510 IU/mL and were titred using polysterene microtitre plates coated with 10 μg/mL immunoglobulin. The conjugate titres correspondingly ranged from 1:100 to 1:500 depending on the enzymatic activity. Retention of the original enzymatic activity ranged from 16-65%. Passage of the conjugates through Sephacryl S-300 superfine gel filtration helped remove unconjugated enzyme and immunoglobulin from the conjugate. The alkaline phosphatase conjugates were used to titrate Coxsackieviruses B1-B6 using checkerboard titration systems. A positive test value was taken as two times the absorbance of the normal HeLa cell control provided the latter value was equal to or less than 0.150

units. The optimal antigen concentration was at a 10^{-3} dilution of the infected HeLa cell cultures except for Coxsackievirus B3 which reacted optimally at a 10^{-2} dilution. No reaction was evident in any of the control wells and their absorbances were zero. The antisera titres ranged from 1:80 to 1:1280 and these endpoints were clearly defined. Secondary experiments employing Coxsackie B-virus antigens in AGMK cell culture resulted in gross non-specific findings which were attributed to non-specific protein binding of the polysterene plates. Preliminary testing of the plates using 1% bovine albumin in the diluents employed during the assay failed to reduce the non-specificity of the plastics and work is ongoing to solve the problem. The ELISA has shown applicability to Coxsackie B-virus detection and, likewise, should be an efficient method of detecting Western Equine Encephalomyelitis virus once the parameters of testing have been completely worked out.

ACKNOWLEDGEMENTS

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INTRODUCTION

The enzyme immunosorbent assay (EIA) has become one of the most promising rapid diagnostic tools for the detection of viral antigens and viral-specific antibodies. From its early conception in the fields of cytochemistry and histochemistry [1], EIA has progressed to a highly specific and sensitive test which rivals the effectiveness of radioimmunoassay in some systems.

Engvall et al. [2] have investigated the parameters of the one-step glutaraldehyde procedure for conjugating alkaline phosphatase (ALP) to immunoglobulins (IgG). The glutaraldehyde molecule forms a cross-link between the ALP and the IgG molecule by binding via one of its two aldehyde groups to amino functional groups on each of the two proteins. A tenfold increase in the IgG to ALP ratio did not improve the conjugate produced and conjugates with the greatest specific activity were attained when a 1:3 IgG to ALP ratio with a 0.2% glutaraldehyde concentration in the reaction mixture was used.

The periodate method of conjugation described by Nakane and Kawaoi [3] results in conjugates with high enzymatic and immunological activity by exploiting the carbohydrate moiety of horseradish peroxidase (HRP) for use in the conjugation reaction. The effects of periodate concentration and of length of exposure to oxidation on HRP were studied and a periodate concentration between 0.04M and 0.08M for a period of thirty minutes was found to produce the optimum conjugate in terms of biological activity. The enzyme to protein ratio in these experiments was 1:1.

Both conjugation procedures have been modified by researchers to accommodate their individual needs in a particular system. The basic mechanisms of covalent binding remain the same in these procedures but the characteristics of the conjugates differ greatly. As yet, there is no recognized standard procedure for either the periodate or the one-step glutaraldehyde method. In this study, conjugates prepared by both methods will be used to investigate the technical aspects of the performance of the indirect microplate ELISA test. The immune sera produced in animal hosts will be purified in preparation for conjugation to the enzymes. The virus antigens used to coat the solid phase will be Coxsackieviruses B1-B6 and Western Equine Encephalomyelitis (WEE) virus. The increased implication of Group B Coxsackieviruses in the etiology of cardiac disease [4] prompted the development of immunofluorescent techniques to detect the viral antigens in tissue culture cells [5,6] and human tissue [7,8]; however these techniques have failed to gain routine application in clinical laboratories. To date, investigation of the ELISA for detecting Coxsackieviruses B1-B6 or specific antibodies has been limited. Techniques have been restricted to detection of the viral antigen in tissue culture [9,10] or to assays employing conjugates prepared by commercial companies [11,12]. In all cases, homotypic reactions resulted in the highest titres; however, low levels of reaction were evident with cross-reacting heterotypic antibodies. The WEE virus presents an extra challenge in the development of an ELISA test for antigen detection in arthropod and animal tissues. A simple, reliable ELISA test for use in epidemiological field studies would be valuable and, as yet, no such procedure has been described for

WEE virus.

The advantage of ELISA tests over other serological tests is that they directly measure the primary interaction between antigen and antibody and are not dependent on secondary phenomena such as precipitation, agglutination or complement fixation. The major drawback of the ELISA test is the lack of standardized protocols for conjugate preparation and titration as well as performance of the assay itself. An attempt to provide guidelines for alkaline phosphatase and horseradish peroxidase conjugate preparation for use in microtitre ELISA will be made in this study. Limits of positivity and negativity will be investigated and the applicability of the ELISA test for detection of Coxsackie B and WEE viruses will be evaluated. Problems encountered in producing the ELISA reaction and their possible solutions will also be explained and discussed.

Chapter I

LITERATURE REVIEW

The measurement of substances in biological fluids has been greatly enhanced through the use of assay systems which use antigens, haptens or antibodies labelled with an enzyme. A variety of names such as enzyme-immunoassay (EIA), enzyme-linked immunoassay, enzyme-coupled immunoassay, immunoenzymatic assay and enzyme-linked immunosorbent assay (ELISA) have been given to the technique which are basically synonymous. EIA and ELISA refer to the same technique except EIA has broader connotations and may refer to the assay of antibody or antigen while ELISA usually denotes a specific assay for a particular antibody.

EIA belongs to the group of binding assays which depends on the recognition properties of antibodies for their antigens. The most commonly used label in the group is a radioisotope (RIA) [13], although enzymes (EIA) [14], erythrocytes (HAI) [15], bacteriophages (virioimmunoassay) [16], fluorescent groups (fluoroimmunoassay) [6] and stable free radicals (spin immunoassay) [17] have all been used. In all cases, the amount of labelled antigen or antibody bound depends on the concentrations of the other components of the system. If one of the components is changed, the distribution of the labelled element between the bound and unbound fractions is altered. The importance of titrating each individual component for each system becomes of paramount importance in order to obtain accurate and reproducible results [18,19]. The actual performance of these techniques also dictates the reliability of the results. In the case of EIA, no standardized procedure for the test has been elucidated which applies to all systems. Attempts have been made to provide routine guidelines; however, none of these hold true in every case [20,21,22]. The distribution of the label is measured by exploiting its properties whether development be fluorometric, colorimetric or radiometric.

The development of EIA grew out of the use of enzyme-labelled antigens and antibodies in histochemistry and cytochemistry [1,18,23]. The resultant technique had several distinct advantages:

1. elimination of the need for radioactive substances
2. reagents are relatively inexpensive and have long shelf-lives
3. assay is simple and inexpensive
4. method is fast and easy to perform
5. technique has wide applicability
6. results are sensitive and specific
7. method is adaptable for automation
8. results may be read using either a light or electron microscope or with the naked eye.

As in most immunoassays, various systems have been developed and reviewed [24,25,26,27]. EIA systems can be devised to measure antibody or antigen using different designs. These systems may be classified in the following ways:

1. on the basis of which reactant is to be detected
2. on the basis of which reactant is labelled
3. by the nature of the reaction, i.e. competitive vs. non-competitive

4. on whether or not the antibody-bound and free reactant are to be separated.

For this reason terminology in the literature becomes very confusing, and the classification of systems used in this paper will be explained.

The competitive EIA was the first system used and was styled after competitive RIA [2,28,29]. The major setback was finding a way to separate the bound from the unbound antigen in order to quantitate one fraction or the other. Two systems stemmed from this need:

1. Homogeneous EIA - where the assay depends on the inhibition of enzymatic activity when the labelled macromolecule is immunologically bound, therefore eliminating the need for a separation procedure.
2. Heterogeneous EIA - wherein a solid phase antigen or antibody allows separation of bound from free reactant for subsequent quantitation [31].

The homogeneous system is applicable exclusively to assays using haptens which possess limited molecular sizes; consequently, the focus of current EIA procedures has been of the heterogeneous type.

If the presence of *antigen* is to be measured, the simplest system is the direct method (also known as the sandwich method) which involves the use of one antibody. Antibody is used to coat the solid phase. The antigen is then added and, after incubation, the excess antigen is washed away. Subsequently added enzyme-labelled antibody binds to the antigen retained by the "capture" antibody on the solid phase. Excess labelled antibody is removed and the appropriate substrate is added. The substrate is converted to a visible form by the enzyme which is usually measured by sight or spectrophotometrically. This system has been used for detection of hepatitis A and B viruses [32,33,34,35], human rotavirus [36], cytomegalovirus [37] and some plant viruses (arabic mosaic virus and plum pox virus) [38]. The major disadvantage to the technique is the need for a specific conjugate to detect each antigen. To overcome this expense, the indirect or double antibody EIA was developed. Antibody to the same antigen is produced in two different animal species. Antibody-1 is used to coat the solid phase and added antigen is "trapped". Excess antigen is removed and antibody-2 is added. After removal of excess antibody-2, an antispecies-2 immunoglobulin labelled with an enzyme is added. After washing, the substrate is added and color development occurs. The advantages to the system are the need for only one type of conjugate for many antigens and increased sensitivity [39]. The indirect ELISA has been used for detection of rotavirus [40,41], E. coli enterotoxin [42], Coxsackieviruses A and B [10,11,43] and baculovirus [44].

For the detection of specific *antibody* the most popular procedure appears to be the indirect method. Antibody in the test sample is allowed to bind to excess solid-phase antigen. After washing, enzyme-labelled anti-species immunoglobulin is used to quantitate the amount of antibody in the original sample. Antibodies to a wide variety of microbiological agents have been quantitated using the above method [12,45,46,47,48].

Rather extensive studies performed by Voller et al. [18] lead him to the conclusion that "...--the 'double antibody sandwich' for detection of antigen and the indirect method for the detection of antibody--are the most useful in practice." Comparison of indirect to direct ELISA for detection of baculovirus [49] indicated that the indirect method was more specific and sensitive. An added advantage to the use of the indirect method for antibody quantitation is that it may easily be converted to detect antigens [50,51]. In general, regardless of the system, enzyme-labelling of the antibody rather than the antigen is more convenient since the latter may not be available in a sufficiently purified form [39].

The advantages and drawbacks of different types of EIA will depend on the application and availability of the antigens and antibodies. The advantages of using labelled antibodies are the constancy of the labelling procedure and the availability of the antibody in a pure form. The major drawback is the greater quantity of antibody needed in the assay as compared to antigen-labelled assays [52]. In addition, the use of labelled antibody is subject to interference by rheumatoid factor in virus-specific assays for antibody [53,54,55]. Rheumatoid factor can be detected in many patients with viral disease and, to some extent, in normal individuals.

The quality of any serological test depends largely on the quality of the reagents used in the test. Purification of antigen and antibody results in increased specificity [56]. Isolation of human and rabbit IgG from sera using protein A-sepharose is especially easy and effective [57]; however, one must keep in mind that the fraction of total IgG which binds to Staphylococcal protein A varies from species to species. In the case of murine immunoglobulins, each class of IgG binds to protein A by varying degrees and is subsequently eluted at different pH's [58,59]. Care should be exercised in utilizing protein A-sepharose in order to avoid inadvertent loss of valuable immunological molecules by failure of these molecules to attach to protein A. Goding[60] presents a simple procedure to extract rabbit IgG from whole serum using protein A covalently bound to cross-linked sepharose 4B (Pharmacia). Only a minute amount of IgG was detected in the effluent after the serum had passed through the column and no contamination of the immunoglobulin with extraneous protein was noted in the elution profile. Preliminary precipitation of IgG before passage through protein A-sepharose is helpful in avoiding clogging of the column; however, as long as the binding capacity of the column is not surpassed, serum may be applied directly.

Purification of antigens used in coating ELISA plates is necessary in order to reduce nonspecificity. The problem of antigen purification becomes difficult as the researcher is often dealing with a highly heterogeneous population of antigenic determinants. Indeed, satisfactory results may be obtained using absolutely no purification procedure [61], although most workers tend to partially purify their antigens. Purification methods for viral antigens range from simple ultracentrifugation [62] or sonication [63], to extraction procedures using fluorocarbons [21,64], polyethylene glycol [14,65], ether [14,66], sucrose-acetone [67] or protamine sulfate-ethanol [68], to more sophisticated procedures employing sucrose or cesium chloride gradient ultracentrifugation [11,14,69] and anion-exchange chromatography [14]. Quantitation of resultant antigen concentrations can be made biochemically (i.e., protein content); however, these values give no indication of biological activity. Each antigen must be titred through checkerboard titratation with the corresponding antibody in the actual ELISA test [20].

Many enzymes such as peroxidase, alkaline phosphatase, glucose oxidase, malate dehydrogenase, glucose-6-phosphate dehydrogenase, acetylcholinesterase, glucoamylase, lysozyme, and Beta-galactosidase have been used as enzyme labels. Enzymes used for labelling should ideally meet the following criteria:

1. be available cheaply and in pure forms
2. have high specific activities
3. retain their specific activities after conjugation
4. be stable during the test procedure
5. have long shelf-lives
6. be absent from biological fluids and antigenic materials being tested
7. have a stable color product.

Horseradish peroxidase (HRP) and alkaline phosphatase (ALP) are the most commonly used enzyme labels. HRP is a hemoprotein with a molecular weight of 40,000 daltons of which approximately 18% is due to a carbohydrate moiety. The function of HRP is to remove highly reactive inorganic compounds of oxygen of intermediate oxidation states between those of oxygen and water (i.e., superoxide anions and H_2O_2). The proposed enzymatic cycle for HRP [70] is that indicated in Fig. 1. The structures of HRP-I and HRP-II are independent of the oxidizing agent used in their preparation and their spectra are identical. Commercial preparations of HRP may contain a homogeneous population of HRP molecules or may have a mixture of up to seven different isoenzymes, thus causing large deviations in characteristics from batch to batch.

Alkaline phosphatase, on the other hand, is a zinc metalloprotein with a molecular weight of 100,000 daltons [71]. Its function is to catalyze the splitting of phosphomonoesters to the corresponding alcohol, phenol or sugar with the release of inorganic phosphate. In addition, ALP possesses a transferase activity enabling it to transfer

$$\text{HRP (native)} + H_2O_2 \rightarrow \text{HRP-I}$$

$$\text{HRP-I} + AH_2 \rightarrow \text{HRP-II} + AH$$

$$\text{HRP-II} + AH_2 \rightarrow \text{HRP} + AH$$

Fig. 1 Proposed enzymatic cycle for horseradish peroxidase.

the terminal phosphate group from a phosphate ester to an acceptor alcohol. The presence of magnesium ions enhances the activity of ALP. The major source of ALP is calf intestine, the most active enzyme being found at the surface of the microvilli.

Each of the above enzymes has its own advantages which may render it more suitable for a particular system [72,73]. On the other hand, two enzymes may be equally qualified as labels for a given system [21,74,75]. Measurement of the label is usually achieved by visible or ultraviolet photometry, occasionally by the naked eye and, most recently, by fluorimetry [76,77,78,79].

Conjugation of enzyme to protein involves the use of a cross-linking agent. High enzymatic and immunological activity as well as stability are desirable features in a conjugate. The cross-linking agent must have at least two active groups in order to link enzymes to proteins. Many chemicals have been used including p,p'-difluoro-m,m'-dinitrophenyl sulfone [80], cyanuric chloride [81], toluene diisocyanate [82], tetra-azotized-o-dianisidine [81], water-soluble carbodiimides [83], N,N'-o-phenylenedimaleimide, sodium-m-periodate [3], glutaraldehyde [75] and p-benzoquinone [84]. Only periodate and glutaraldehyde were used in the following experiments, and further discussion will be limited to these two agents.

There are two basic theories concerning the conjugation procedures: the one-step and the two-step methods [75,85]. The one-step method involves suspending the protein and enzyme together in solution before adding the bifunctional reagent. The resultant conjugate consists of a heterogeneous population of high and low molecular weight protein/enzyme macrocomplexes. This method is popular because of its simplicity. The two-step method combines the enzyme with the cross-linking agent first. Excess reagent is removed and the protein is added. The degree of self-linking of the immunoglobulin is reduced using this technique, resulting in a conjugate which is more homogeneous in nature.

Periodate

The low coupling efficiency found with other bifunctional reagents was thought to be due to the blockage of the majority (up to as much as 99.5%) of α- and ε-amino groups and some hydroxy groups by allylisothiocyanate found in HRP preparations. The periodate method of conjugation (Fig. 2) by-passes these amino groups and uses the carbohydrate moiety of HRP [3,86]. Approximately 70% of the HRP couples with protein and about 99% of the protein will be labelled with HRP. No significant loss in IgG immunologic activity or enzymatic activity was reported by Nakane and Kawaoi [3]. Other workers have found that immunologic and enzymatic activities are reduced to 10-17% and 25-30% respectively [87,88]. Yamashita and his workers [89] found periodate HRP conjugates purified by DEAE-cellulose chromatography retained 50% of conjugate immunoreactivity. The low immunological reactivity in periodate

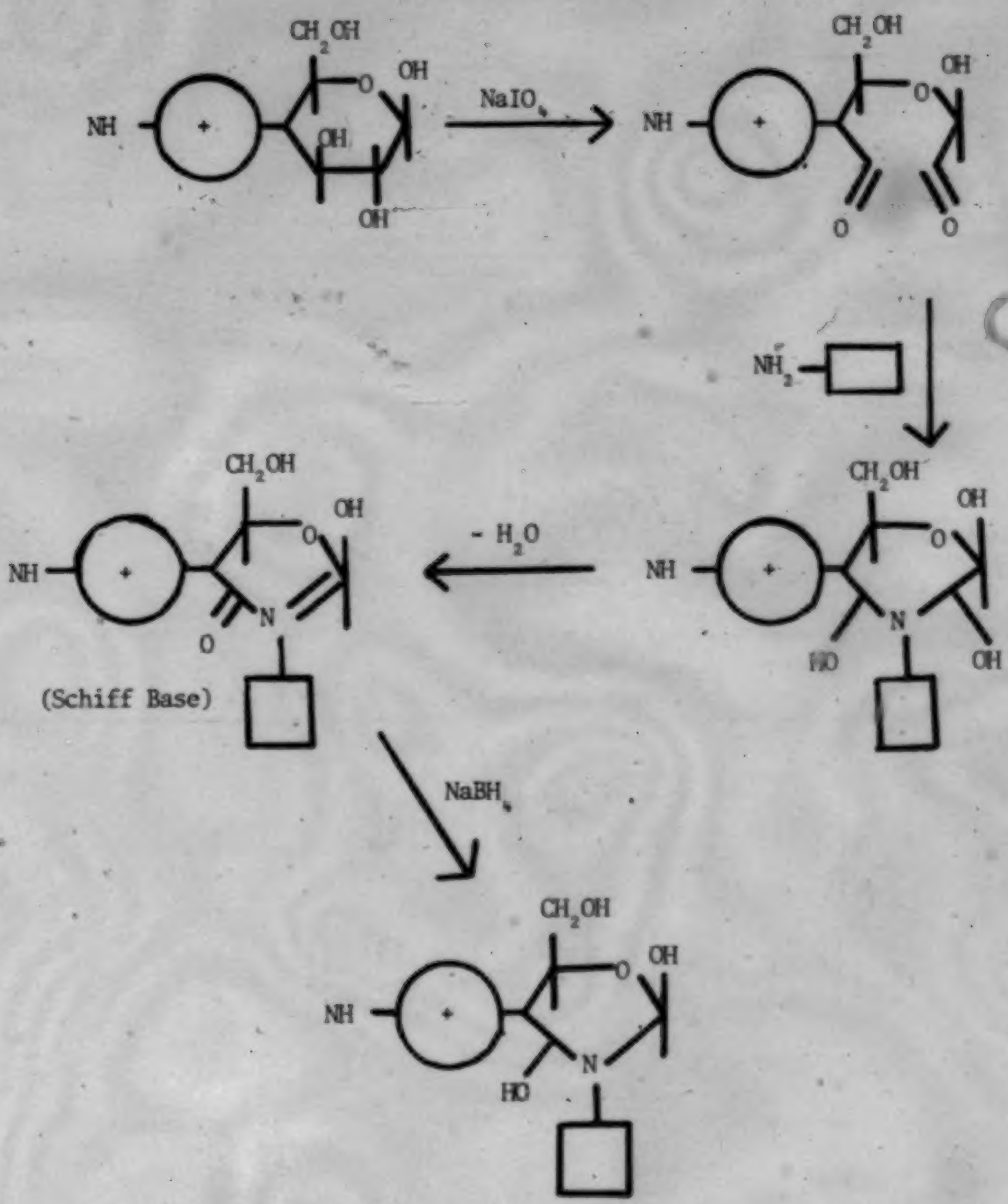

CH2OH
OH
O
NH
OH
OH
NaIO4
CH2OH
OH
O
NH
O
O
NH2
CH2OH
OH
O
NH
N
HO
OH
- H2O
CH2OH
OH
O
NH
N
O
(Schiff Base)
NaBH4
CH2OH
OH
O
NH
N
HO

Fig. 2 HRP-IgG coupling mechanism using periodate.

conjugates may be due to the addition of sodium borohydride as indicated by Saunders (G. C. Saunders, unpublished results, 1978), who found a 50-60% increase in activity when this step was omitted [56]. The conjugate is highly heterogeneous and the molecules have a molecular weight of over 400,000 daltons which elute in the void column during gel filtration [90,91]. Complete separation of conjugated immunoglobulin from free immunoglobulin is impossible using Sephadex G-200 but can be achieved using Ultrogel AcA-44 or Sephacryl S-300 polyacrylamide gels [90]. Macromolecules tend to adsorb nonspecifically resulting in high background reactions which limit the sensitivity of the conjugates in EIA unless this aspect is accounted for and corrected [92].

Glutaraldehyde

Glutaraldehyde is water soluble and, therefore, more reactive than difluoro-dinitrophenyl sulfone as a cross-linking agent (Fig. 3). Conjugation by the one-step procedure produces extensive self-coupling of the immunoglobulin (IgG) molecules, drastically reducing the immunoreactivity of the conjugate. Sedimentation studies indicate that although these conjugates are highly heterogeneous in nature, the IgG macrocomplexes are predominantly trimeric with a molecular weight of about 600,000 daltons [75,82,90]. Most of the IgG is linked to horseradish peroxidase although less than 5% of the enzyme is conjugated retaining 60-70% of its original specific activity [82,90]. The low reactivity of the HRP is probably due to the blockage of its amino groups by allylisothiocyanate [3]. Separation of unconjugated, self-coupled IgG from conjugated IgG is unnecessary since virtually all IgG

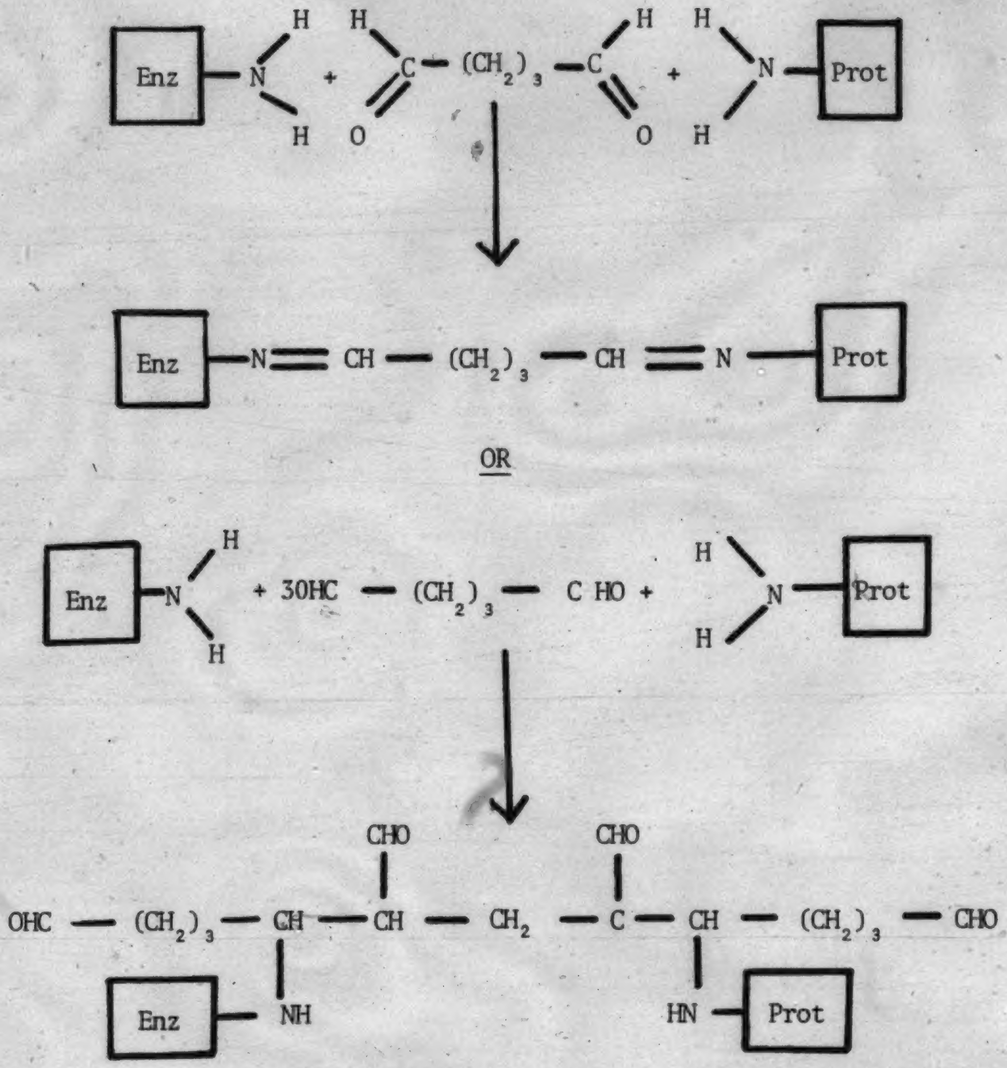

Enz
H H
N + C — (CH2)3 — C + N
H O
O H
Prot
Enz — N = CH — (CH2)3 — CH = N — Prot
OR
Enz — N H H + 3OHC — (CH2)3 — CHO + H H N — Prot
CHO CHO
OHC — (CH2)3 — CH — CH — CH2 — C — CH — (CH2)3 — CHO
Enz — NH
HN — Prot

Fig. 3 Possible mechanisms for glutaraldehyde conjugation.

is labelled. Unconjugated, monomeric IgG is easily removed by gel filtration chromatography [25]. Such conjugates may be used in immunocytochemistry, although the IgG aggregates most likely increase the nonspecificity of interaction.

Conjugates prepared by the two-step glutaraldehyde method are homogeneous in nature, consisting of one molecule of IgG bound to one molecule of enzyme (MW of 190,000 daltons) [85,30,93]. Polymerization of IgG is eliminated as IgG is not exposed to uncombined glutaraldehyde and no intramolecular bonds can occur. The small number of amino groups on the peroxidase molecules limits intramolecular bonding and glutaraldehyde reacts with the enzyme via one arm only leaving the other free to react with immunoglobulin. Polymers may form if an HRP molecule binds to two IgG molecules or vice versa; however, this rarely occurs due to the strikingly low percentage of HRP actually partaking in linkage reactions. Forty percent of Fab or antibody added and 3-5% of peroxidase added become coupled [85,94]. Immunological and enzymatic activity retained is 50% and 50-75% respectively of the original activity [90,93,95]. Other workers [96,97] have obtained conjugates which indicate only 2-10% of the IgG becomes conjugated with half of its original immunoreactivity retained but less than 1% of HRP activity remained after conjugation to the IgG (Kuijpers, L. P., Wolters, G. and Schuurs, A. H., unpublished data). In comparison, conjugates produced using AP and IgG retain 1-10% of antibody activity and 60-70% of enzymatic activity of which roughly half is actually linked to immunoreactive IgG [2,98].

All types of EIA (except homogeneous EIA) require separation of free from bound labelled molecules. The method used should be fast, reproducible and harmless to the conjugate. Incorporation of a solid phase into the system has been widely used since separation is achieved by a simple washing procedure. Sepharose, sephadex, paper and cellulose are usually activated using cyanogen bromide (available commercially) for coupling antigens or antibodies to their surfaces [72,81]. More convenient solid phases rely on passive adsorption of proteins to plastics as first discovered by Catt and Tregear [99]. Since then, numerous plastics such as cellulose nitrate, polyallomer, polystyrene and polyvinyl have been examined [100]. These plastics have been formed into beads [69,101], microcuvettes [102], microtitre plates [18] or, most recently, "sticks" [103,104] for use in EIA.

Nonspecific adsorption of immunoreagents added subsequent to the coating procedure has been reduced by addition of protein solutions plus a non-ionic detergent to buffered diluents used during the testing procedure [45,66]. An inherent difficulty in the use of these inhibitors is increased desorption of the protein coated on the solid phase in their presence. The effect is minimal at coating concentrations of 2-10 μg antibody protein/mL according to Herrmann et al. [66] and only becomes of concern when high concentrations (100 μg/mL) are used. In contrast, Engvall et al. [2] recorded a 40% desorption of antibody even at the lower concentrations. Evidently, each system must be examined as to the extent to which this factor may interfere with the test. In an effort to circumvent the problem, the solid phase may be pretreated with poly-L-lysine to covalently bind the protein [66,105]. The most significant reduction in nonspecific adsorption of immunoreagents occurs when both inhibitors (usually bovine serum albumin and Tween-20) are added to the buffered diluents [66]. Post-treatment of the solid phase, following the coating procedure, with a protein solution may reduce nonspecificity by saturating all remaining protein adsorption sites. Some investigators use as much as a 5% solution of bovine serum albumin or normal sheep serum for this purpose [77,106,107]. Nylon has been used most recently as a solid phase, the advantage being that the antibodies are immobilized by covalent linkage using glutaraldehyde or carbodiimides; hence, nonspecific adsorption and antibody desorption are eliminated [108].

Enzyme immunoassays have been compared in various different systems to the classic techniques of hemagglutination inhibition, immunofluorescence, neutralization, complement fixation and radioimmunoassay. When EIA is compared to HAI tests, results are extremely contradictory. Parker reported that ELISA was 300 times more sensitive than HAI tests for detecting Sendai virus in mice [63], Shekarchi et al. found comparable ability in detecting positive and negative sera for rubella virus infection between the two tests [109] and Hammond et al. claimed that ELISA was less sensitive than HAI for serodiagnosis of influenza A virus [14]. Upon comparison of EIA to CF testing, the consensus was that EIA was more sensitive from anywhere between 16 and 100 times [14,12,63,64,100]. Slight contradiction was evident when neutralization testing and EIA were compared. In some cases, EIA was reported as being more sensitive than the NT [77,111], while others found the two tests were equal in degree of sensitivity [12,62,112]. The replacement of fluorescein with peroxidase as a label in staining tissue culture cells has also prompted many parallel studies using the two types of conjugates. In most instances, peroxidase conjugates appeared to be slightly more sensitive than fluorescein conjugates [9,110,113], although equal sensitivity has been noted in cell counting techniques [114]. At one time, ELISA was promoted as being a replacement test for radioimmunoassays. Some researchers found the ELISA just as sensitive, specific and reliable as RIA, provided proper confirmatory tests (blocking ELISA's) were included [32,69]. Hillyer et al. stated that in his system, RIA had a diagnostic sensitivity of 95% as compared to only 75% sensitivity using the ELISA [115]. Indeed, the ELISA may be the test of choice for some assays; however, quantitation of minute amounts of drugs, steroids or hormones (i.e., in the femtomole range) requires RIA as the only method sensitive enough to detect these levels [116]. The above comparisons were made in numerous completely different systems which must be kept in mind when looking for any specific trends.

Two of the most attractive aspects of ELISA are that many of the conjugates are available commercially in kits or separately and, secondly, a relatively short amount of time is needed to perform the test. The ELISA has been adapted to a wide variety of biological systems as reviewed, and is currently being adapted to many more through various modifications. To date, the test is being used actively for clinical diagnosis as well as a research tool, although a standardized technique has not yet been developed.

Chapter II

HORSERADISH PEROXIDASE

Our initial intent was to detect specific antigens in tissue cultures infected with Coxsackieviruses B1-B6 by staining with an enzyme conjugate. Horseradish peroxidase seemed the enzyme of choice because of its small molecular weight (40,000 daltons) and its relatively low cost. Once conjugated to immunoglobulin, the complex has a molecular weight of roughly 190,000 daltons.

Preparations of HRP differ greatly between batches; therefore, a preparation with the highest purity was desired. The purity of HRP is measured by its R.Z. factor which is equal to the absorbance of the solution at 403 nm divided by its absorbance at 280 nm. This value denotes the ratio of hemin to protein content. Pure HRP has an R.Z. factor of 3.0.

A. PURIFICATION OF THE ENZYME BY COLUMN CHROMATOGRAPHY

Type II and Type VI HRP were purified by column chromatography in:

a. a Sephadex G-100 superfine column (Type II and Type VI HRP) and

b. a Sephacryl S-300 superfine column (Type VI only),

in order to assess whether or not it was economically advantageous to employ Type II HRP for conjugation as it is much less expensive than Type VI HRP.

1. Type II HRP (Sigma)

One hundred milligrams of Type II HRP (R.Z. = 1.5) were dissolved in 2.5 mL of Tris-HCL (0.1 M) + NaCl (0.2 M), pH 8.0 at 4°C. The entire sample was placed on a 90 cm × 1.6 cm Sephadex G-100 superfine column. The void volume was 65.0 mL as determined using 0.2% blue dextran. Fractions 18-27 (Fig. 4) were pooled and collected since these tubes contained the major peak. A 1:10 dilution of the pool was read in a Beckman M-25 scanning spectrophotometer, against the buffer, to determine the R.Z. value.

$$\text{Absorbance (Abs.) at 280 nm} = 0.393$$

$$\text{Abs. at 403 nm} = 0.900$$

$$\text{R.Z.} = \frac{0.900}{0.393} = 2.29$$

The pool was then scanned from wavelengths 350-750 nm. Two extra peaks, one at 490 nm and the other at 635 nm, besides the major peak at 403 nm, were evident. The final total protein content was 54 mg (only 54% recovery) as determined by the Biuret method [117].

The presence of the two extraneous peaks prompted us to attempt a second purification. The collected sample was first concentrated to 4 mL using an Amicon concentration cell (PM-30 membrane) and was repassed through the column. The results (Fig. 5) showed a tiny peak followed by the HRP peak. Fractions under the HRP peak were collected and dialized against deionized water. The R.Z. value was 3.0, indicating that a high degree of purity had been achieved. When the total protein content was determined, only 15 mg of protein remained, indicating that although passage through two successive Sephadex G-100 superfine

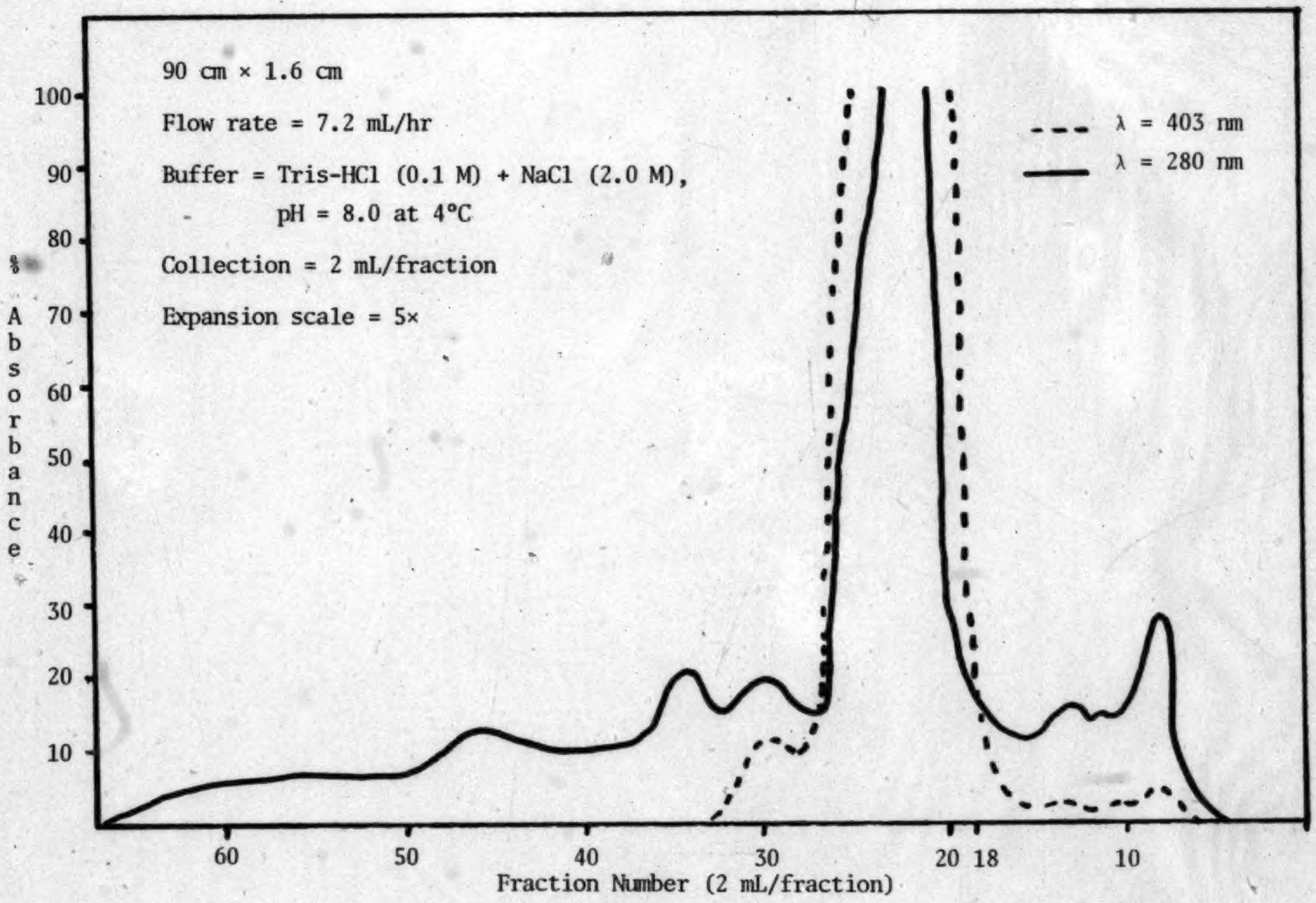


Fig. 4 Type II HRP (Sigma) 1st passage through Sephadex G-100.

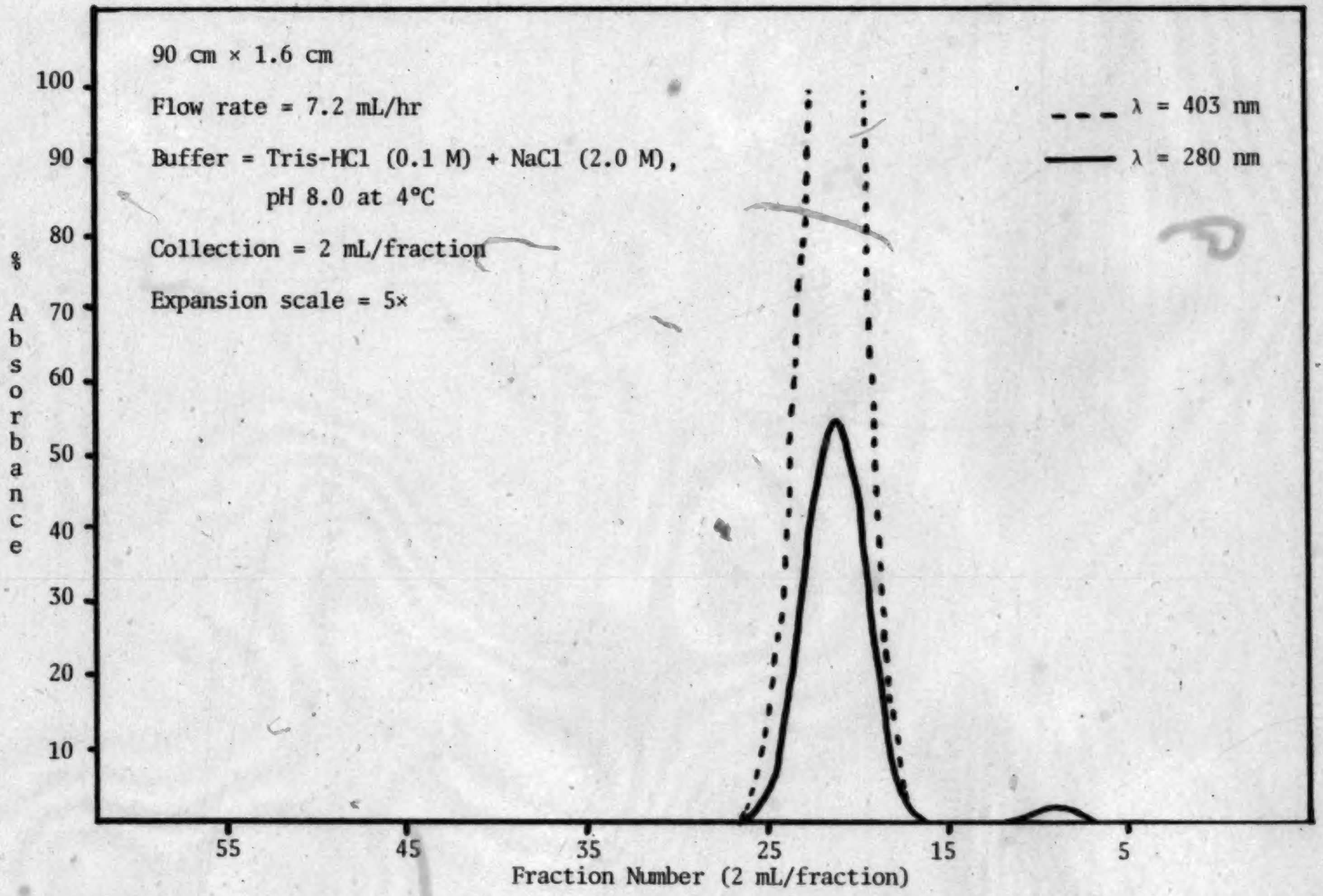


Fig. 5 Type II HRP (Sigma) 2nd passage through Sephadex G-100.

columns resulted in satisfactory purification of Type II HRP, the small amount of protein recovered (15%) makes this purification procedure unfeasible.

2. Type VI HRP (Sigma)

Fifty milligrams of Type VI HRP were dissolved in 2.5 mL of Tris-HCl (1.0 M) + NaCl (0.2 M), pH 8.0 at 4°C. The solution was placed on the same (Sephadex G-100 superfine) column as previously mentioned for the Type II HRP. No extraneous peaks were evident (Fig. 6) and the enzyme preparation was considered to be pure (given R.Z. = 3.0).

For comparison purposes, we decided to pass the Type VI HRP through a Sephacryl S-300 superfine column (Fig. 7), as it was a new dextran-acrylamide gel boasting excellent physical, chemical and thermal stability (pamphlet from Pharmacia). Ninety-one milligrams of Type VI HRP in 2.5 mL of Tris-HCl buffer, as previously used, were applied to a 55 cm × 1.6 cm column of Sephacryl S-300 superfine (void volume = approximately 41 mL). While there was no difference between the Sephacryl S-300 and the Sephadex G-100 profiles, the advantages in using the Sephacryl S-300 column were:

a. ease of packing the column
b. gel is packaged preswollen
c. column does not overpack with continued use; therefore, columns last for at least 6 months
d. gel can be autoclaved if necessary
e. an accelerated flow rate can be used, decreasing the time needed for each run.

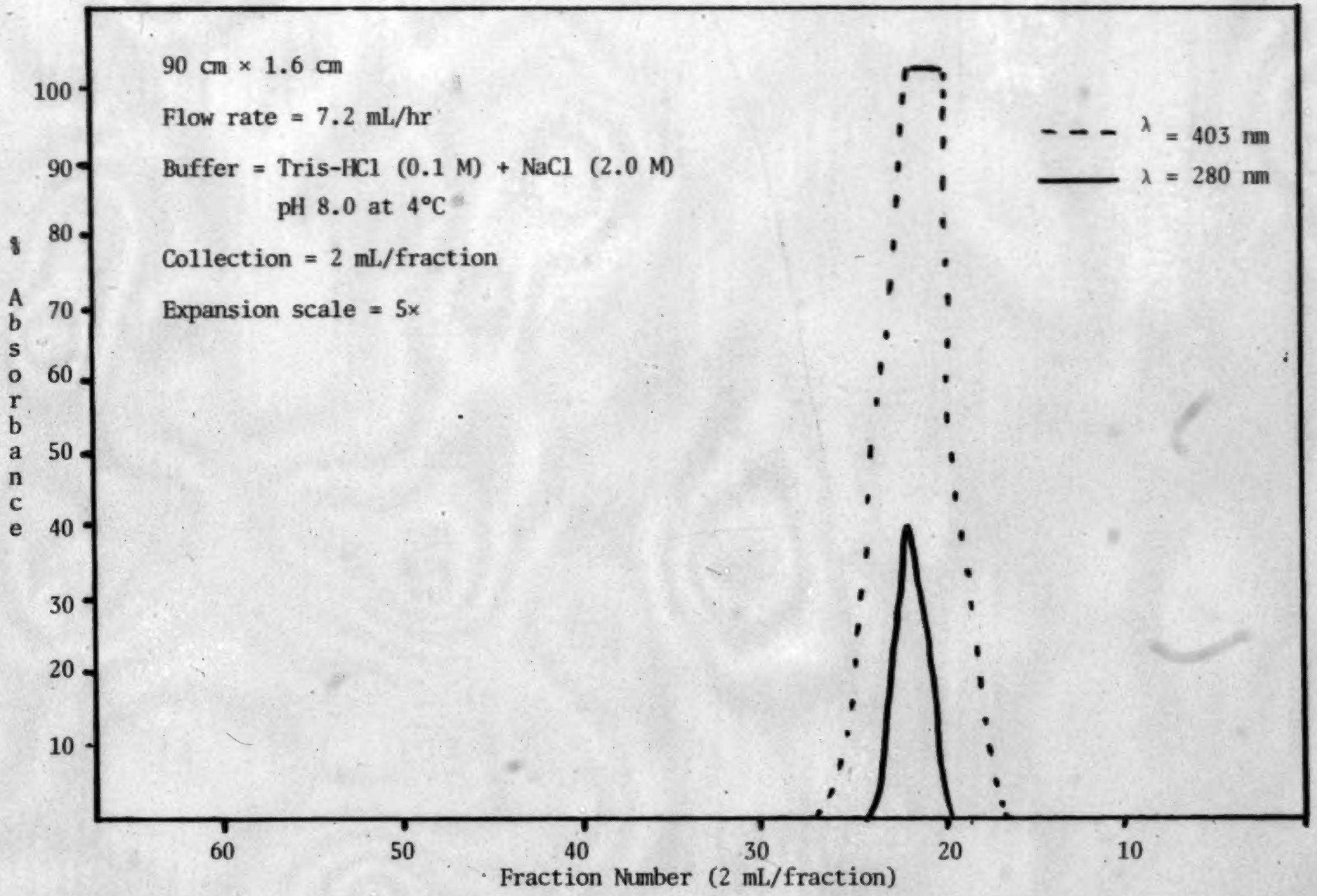


Fig. 6 Type VI HRP (Sigma) passage through Sephadex G-100.

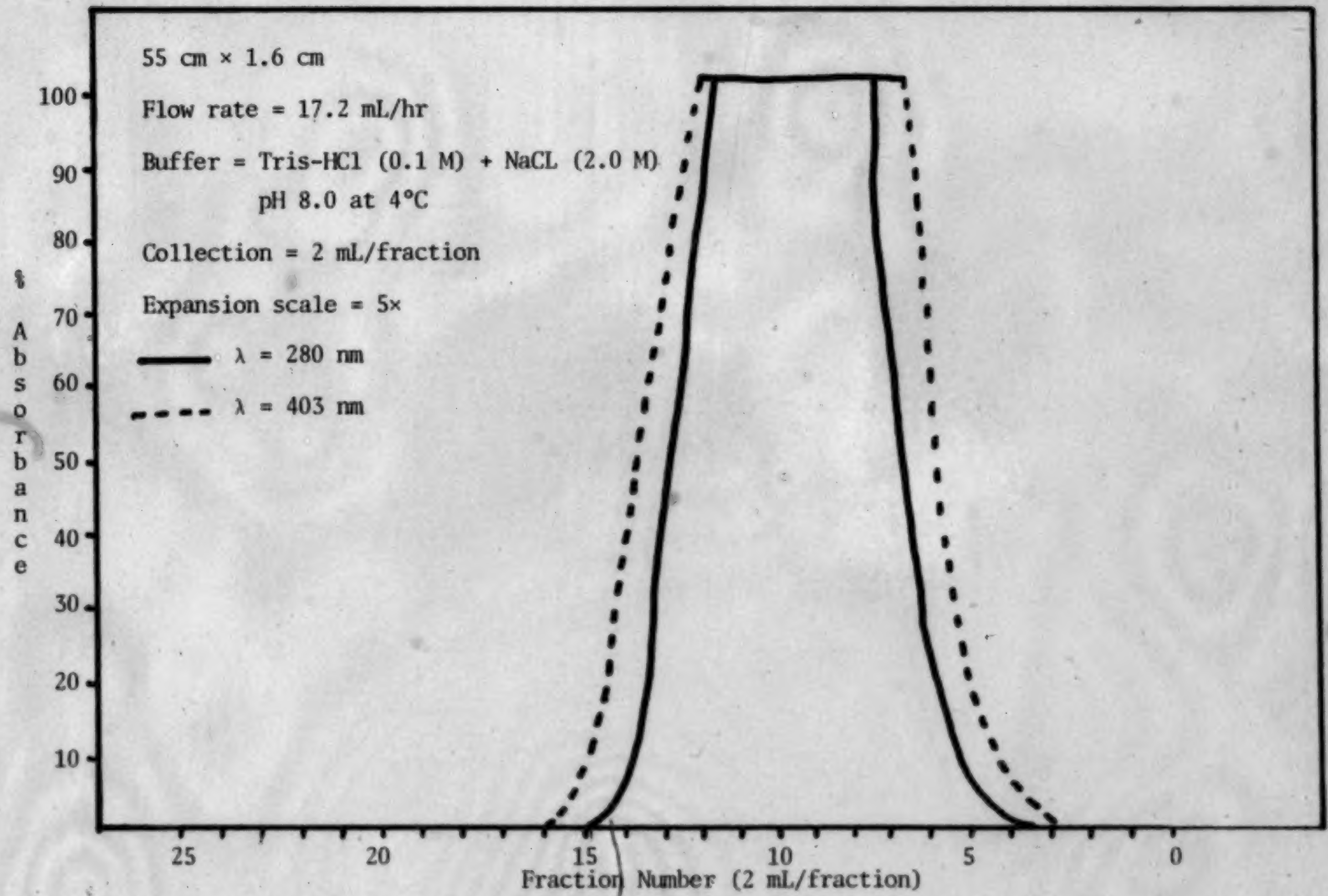


Fig. 7. Type VI HRP (Sigma) passage through Sephacryl S-300.

The only disadvantage of using the Sephacryl S-300 superfine gel was that dextran appeared to partially adsorb to the gel even at pH 8.0, although the Pharmacia company stated it adsorbed only at a pH of less than 6.0. For this reason, determination of the void volume was at best an approximation.

B. ENZYMATIC ACTIVITY DETERMINATION

The enzymatic activity of the purified Type II HRP was determined using o-dianisidine-dihydrochloride (Sigma) in the procedure described by Worthington [118]. The purified enzyme had an enzymatic activity of 262 units/mg. The enzymatic activity given in the Sigma enzyme specifications for highly purified Type VI HRP was 275 units/mg; therefore, our purification of the Type II HRP resulted in a comparatively pure and enzymatically active enzyme.

C. VIRUSES

1. Coxsackieviruses

Coxsackievirus B3 (Nancy) was obtained from Dr. M. H. Hatch, Enteric Virology Unit, National Communicable Disease Center, Atlanta, Georgia. Coxsackievirus B1 (Connecticut 5), Coxsackievirus B2 (Ohio 1), Coxsackievirus B4 (JVB), Coxsackievirus B5 (Faulkner) and Coxsackievirus B6 (Schmitt) were obtained from the Research Reference Reagent Laboratory, National Institute of Health, Bethesda, Maryland.

2. Tissue Culture Cells

The reference strains of Coxsackievirus were passaged once in AGMK cells obtained from Flow Laboratories. Each strain was then passaged three times in HeLa cells also obtained from Flow Laboratories.

Antigen titrations, neutralization tests and normal controls were performed in HeLa culture cells.

3. Tissue Culture Media

The growth medium for HeLa cells consisted of Eagle's minimum essential medium (MEM, Flow Laboratories) supplemented with 5% fetal calf serum, 100 IU/mL of penicillin G, 100 μg/mL of gentamicin and 100 μg/mL of streptomycin and 15 IU/mL of mycostatin. The medium was adjusted to pH 7.4 with 7.5% sodium bicarbonate.

For the AGMK cells we employed Eagle's MEM supplemented with 2% fetal calf serum, 0.5% lactalbumin hydrolysate (Grand Island Biological Co., Inc.) plus antibiotics.

The maintenance medium used in both cases was equivalent to the growth medium without the calf serum.

4. Propagation of Coxsackieviruses

Monolayers of culture cells were grown in Roux bottles. The growth medium was discarded and 10 mL of virus suspension containing 10^5 $TCID_{50}$/0.1 mL were inoculated into the bottles. The virus was allowed to adsorb for 60 min at 37°C. After the adsorption period, 100 mL of maintenance medium were added. The infected cells were incubated at 37°C until 4+ cytopathic effect (CPE) had occurred (usually after 24 to 48 hours of incubation). The harvested cells were frozen at -70°C and rapidly thawed three times in order to release the virus from cellular material. The cellular material was removed by centrifugation for 1 hr at 4°C in a Servall RC-2 centrifuge (7700×g, SS-34 rotor) and the supernatant was stored at -70°C.

5. Western Equine Encephalomyelitis Virus

The strain of Western Equine Encephalomyelitis (WEE) virus used was isolated by Morgante et al. in 1965 from the brain of an infected horse [119].

6. Propagation of WEE Virus

Infant mice (Swiss-Webster strain) 2-4 days old were inoculated with 0.02 mL of a 10 LD_{50} of virus. After approximately 34 hr when the mice were moribund, they were exsanguinated. Dead animals were discarded. The brains were removed aseptically and were ground to a 20% suspension in Hank's balanced salt solution (HBSS), pH 7.2. Ten percent fetal calf serum and antibiotics were added if the virus was to be used for propagation. Nothing was added if the virus was to be used for preparing immune sera. The brain suspensions were spun at 7700×g for 1 hr and the supernatant fluids were frozen at -70°C.

The WEE virus was also propagated in HeLa cell culture as described for the propagation of Coxsackieviruses (p. 28). The antigen was stored at -70°C.

D. PURIFICATION OF ANTIGEN

Partial purification of viral antigens was affected using a fluorocarbon extraction procedure as outlined by Hamparian et al. [120]. Equal volumes of virus suspension and Genesolv-D (trifluorotrichloroethane, Allied Chemical) were mixed together with Ballotini beads (1 g/10 mL) and were vigorously shaken by hand for 2 min. After allowing the mixture to sit at 4°C overnight, it was centrifuged at 7700×g for 1 hr in a refrigerated Servall RC-2 (SS-34 rotor). The supernatant fluid was stored at -70°C in the presence of 1:10,000 merthiolate.

Normal HeLa cells and suckling mouse brains were treated in exactly the same manner as the virus suspensions to be used for controls.

E. ANTIGEN TITRATION

1. Coxsackieviruses

Each of the partially purified Coxsackieviruses was titrated in HeLa cells. Tenfold titrations of virus were prepared in tissue culture maintenance media. One hundred microlitres of each virus dilution was inoculated into each of four tissue culture tubes. The tubes were incubated at 37°C and were observed for 7 days for CPE. The 50% end-point was determined as described by Reed and Muench [121]. The titre was expressed as $TCID_{50}$ per 0.1 mL virus suspension. The results are tabulated in Table 1.

2. Western Equine Encephalomyelitis Virus

Tenfold dilutions of the partially purified WEE virus were made in Earle's balanced salt solution (EBSS). A 0.02 mL volume of each dilution was inoculated intracerebrally into each of eight suckling mice from 2-4 days old. The mice were observed for mortality over a period of 14 days. The titre was found to be $10^{9.5}$ LD_{50}.

The WEE antigen propagated in HeLa cell culture was titrated in HeLa cell culture as described for the titration of the Coxsackieviruses. The titre of the WEE virus was 10^6 $TCID_{50}$/0.1 mL.

F. HYPERIMMUNE SERA

IgG fraction of anti-mouse IgG prepared in rabbit was purchased from Miles Laboratories, Inc., Elkhart, Ind. (#65-157).

Table 1. Titre of Coxsackieviruses in HeLa cells.

Virus	$TCID_{50}$/0.1 mL
Cox B1	$10^{7.0}$
Cox B2	$10^{7.25}$
Cox B3	$10^{6.5}$
Cox B4	$10^{6.0}$
Cox B5	$10^{7.25}$
Cox B6	$10^{7.25}$

1. Guinea Pigs

Hyperimmune sera to Coxsackieviruses B1-B6 were prepared in guinea pigs by Morgante et al. [6] in preparation for immunofluorescent testing. Half of each batch was preserved for the ELISA test. The sera were prepared as follows.

Female guinea pigs, three to four months of age and weighing 350-450 g, were immunized with antigens prepared in HeLa cells and partially purified with fluorocarbon (trifluorotrichloroethane) in a manner similar to that described by Hamparian et al. [120]. At various intervals, each animal received simultaneous i.p. and s.c. inoculations in the interscapular region, for a total of six injections over a period of 37 days. Various amounts of virus (1-2 mL in the last two injections) were inoculated i.p. and half that amount was inoculated s.c. The first three times only, undiluted virus (~ 10^6 $TCID_{50}$/0.1 mL) was inoculated. Thereafter, an equal volume of Freund's incomplete adjuvant was added to the viral suspension. All animals were bled by cardiac puncture three and one-half weeks after the last injection. Sera were obtained before immunization from each animal and were tested to ensure the absence of Coxsackievirus antibodies prior to immunization. All sera were preserved at -70°C until used.

2. Mouse

WEE virus propagated in suckling mouse brain was used to immunize three-week-old mice in order to avoid the production of anti-species antibody and reduce the degree of nonspecificity due to this factor in the ELISA test. Pretesting was performed prior to inoculation to determine the maximum dosage the mice could tolerate.

A 10^{-1} dilution of WEE virus in mouse brain was prepared in EBSS. The inoculations were scheduled as follows:

	Intrascapular	Intraperitoneal
Initial injection	1.0 mL	
1st week	0.5 mL	0.5 mL
2nd week	0.5 mL	0.5 mL
4th week	0.5 mL	0.5 mL
6th week	Exsanguination	

The mice were exsanguinated by cardiac puncture and the antisera were stored at -70°C.

G. NEUTRALIZATION TESTS

1. Anti-Coxsackievirus Sera

Neutralizing antibody titres were determined using the varying serum-constant virus technique in HeLa culture cells [122]. The concentration of virus used was about 100 $TCID_{50}/0.1$ mL. The highest dilution of serum protecting the cells from the challenge virus was taken as the neutralization titre. In each of the six Coxsackievirus strains, the titre was greater than 1:512.

2. Anti-WEE Sera

Neutralization testing for mouse anti-WEE sera was performed using the constant serum-varying virus procedure [122]. The normal mouse serum gave an LD_{50} of $10^{7.0}$. The LD_{50} of the test serum was 10^{0}; therefore, the anti-WEE sera had a neutralizing capacity of $10^{7.0}$.

H. IMMUNOFLUORESCENCE TITRE

As previously mentioned, the anti-Coxsackievirus sera used in these studies were originally used to develop an immunofluorescence technique by Morgante et al. [6]. Once conjugated to fluorescein isothiocyanate by a reverse dialysis method, the sera were found to have the titres given in Table 2 when used to stain coverslip cultures infected with the corresponding virus. Cross reactivity was found to be negligible.

I. PURIFICATION OF NORMAL ANIMAL SERA

All the following procedures were performed at 4°C. Preparation of buffers and reagents used are given in the appendix.

1. IgG Precipitation with 50% Ammonium Sulfate

Ten mL serum and 10 mL deionized water were placed in a 125-mL Erlenmeyer flask to which a magnetic stir bar was added. The flask was placed on a magnetic stirrer. Twenty mL of saturated $(NH_4)_2SO_4$, adjusted to pH 7.0, were added drop by drop to the diluted serum. The

Table 2. Homotypic and heterotypic fluorescence staining titres of conjugates to group B Coxsackieviruses.

HeLa cells infected with indicated Coxsackievirus serotype	Highest dilution showing staining						
	Normal guinea pig serum	FITC conjugate					
		B1	B2	B3	B4	B5	B6
B1	<5	40*/80†	10	10	10	10	5
B2	<5	<5	160*/320†	10	10	5	5
B3	<5	5	10	80*/160†	10	5	10
B4	<5	10	10	10	80*/160†	5	<5
B5	<5	10	5	5	10	80*/160†	<5
B6	<5	5	5	10	5	5	160*/320†
Uninfected cells	<5	<5	5	5	5	5	5

NOTE. Protein was adjusted to 10 mg/mL prior to each conjugate dilution. Only the reciprocal of each staining titre is shown. FITC = fluorescein isothiocyanate. Except when indicated otherwise, all preparations exhibited a faint, diffuse cytoplasmic fluorescence.

* 3+ or 4+ perinuclear and pinpoint cytoplasmic fluorescence.

† 1+ or 2+ perinuclear and pinpoint cytoplasmic fluorescence.

flask was covered and stored overnight. Next day, the serum was centrifuged at approximately 7700×g for 15 min in a refrigerated Servall RC-2 (SS-34 rotor) centrifuge. The precipitate was redissolved in 7 mL of PBS, pH 7.2 and was dialized in the cold against 4-500 mL changes of PBS over a period of 2 days.

2. Protein A-Sepharose Chromotography

The methodology followed was that outlined by Goding [60]. Unless otherwise stated, all procedures were carried out at 4°C.

One and one-half grams of protein A-sepharose CL-4B (Pharmacia) was dissolved in PBS (0.01 M), pH 7.4 and was left to swell overnight. The gel was poured into a 20 cm × 1.6 cm column and was equilibrated by letting 40 mL of PBS flow through at a rate of 45 mL/hr. Buffer was allowed to drain to the level of the bed support at which time 100 mg of rabbit IgG fraction II (Miles, #82-455) in PBS, pH 7.4 was layered on top. Twenty millilitres of PBS were passed through the column to wash the IgG into the column and to flush any impurities through. The PBS was once again drained to the bed surface. Glycine-HCl, pH 2.8 was added to elute the adsorbed IgG. The fractions were collected until no more protein was eluted. IgG fractions were collected in tubes containing carbonate-bicarbonate buffer, pH 9.3, in order to avoid denaturation of the immunoglobulin by prolonged exposure to acid. Afterwards, the column was re-equilibrated with 40 mL of PBS.

3. Normal Rabbit IgG-Fraction II (Miles)

Figure 8 illustrates the peak obtained after passage of the control rabbit IgG through Protein A-sepharose. The small peak which passed through the column in the PBS indicated the presence of protein

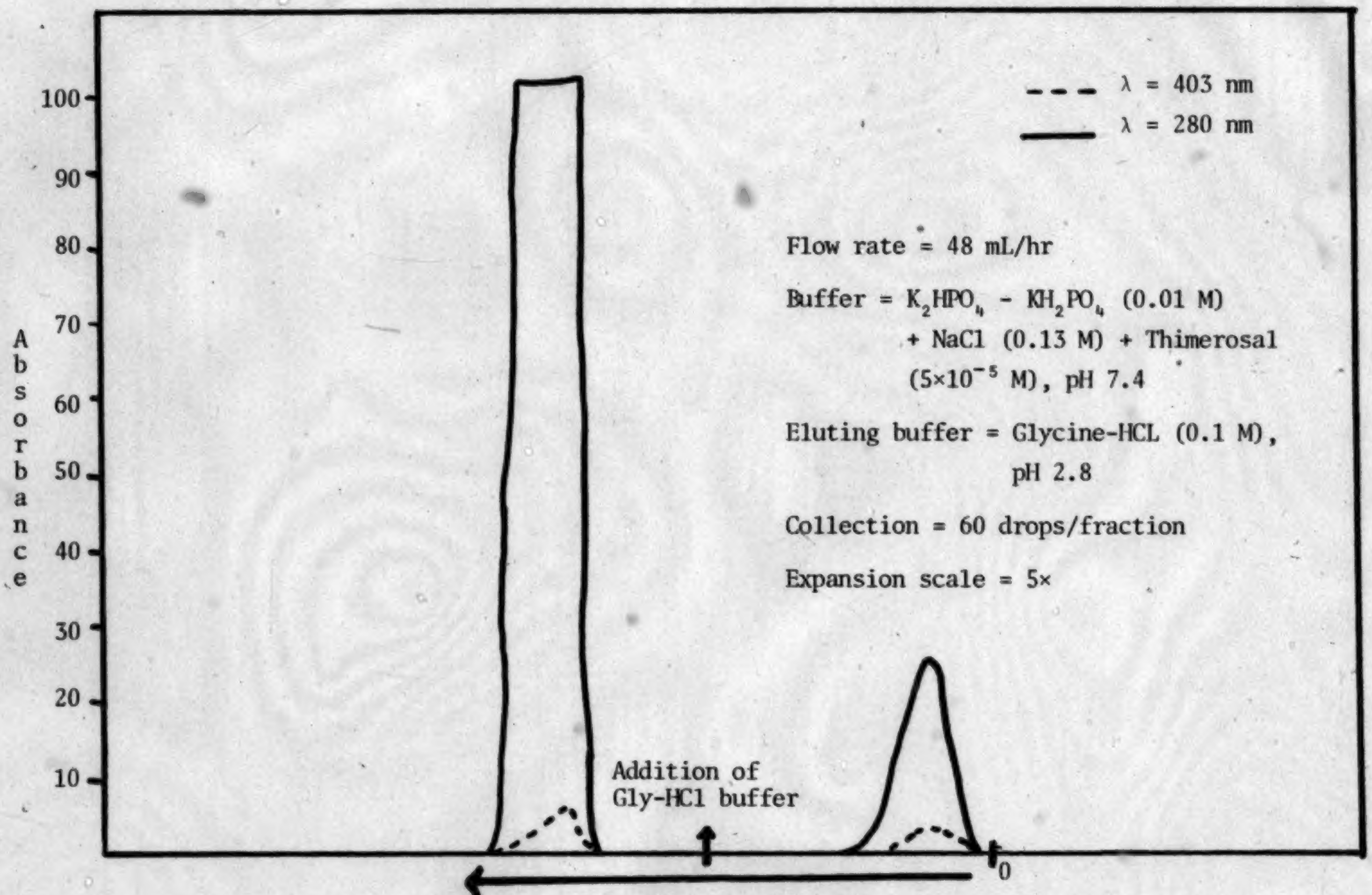


Fig. 8 Normal rabbit IgG passaged through Protein A-Sepharose.

other than immunoreactive IgG which was bound to the protein A-sepharose gel. The small amount of absorbance at 403 nm seen in both peaks may have been due to hemin compounds inherent in the IgG preparation.

4. Anti-mouse IgG Rabbit IgG (Miles)

The effluent protein peak observed after passing the rabbit anti-mouse IgG onto the protein A-sepharose column using PBS was much larger than the one observed in the normal rabbit IgG profile (Fig. 9). In sharp contrast to the normal rabbit IgG, the glycine-HCl-eluted peak of the rabbit anti-mouse IgG showed no absorbance at 403 nm.

5. Mouse Anti-WEE IgG

Anti-WEE sera prepared in mice were purified only by 50% ammonium sulfate precipitation in order to avoid loss of usable immunoglobulins during protein A-sepharose purification [57,58].

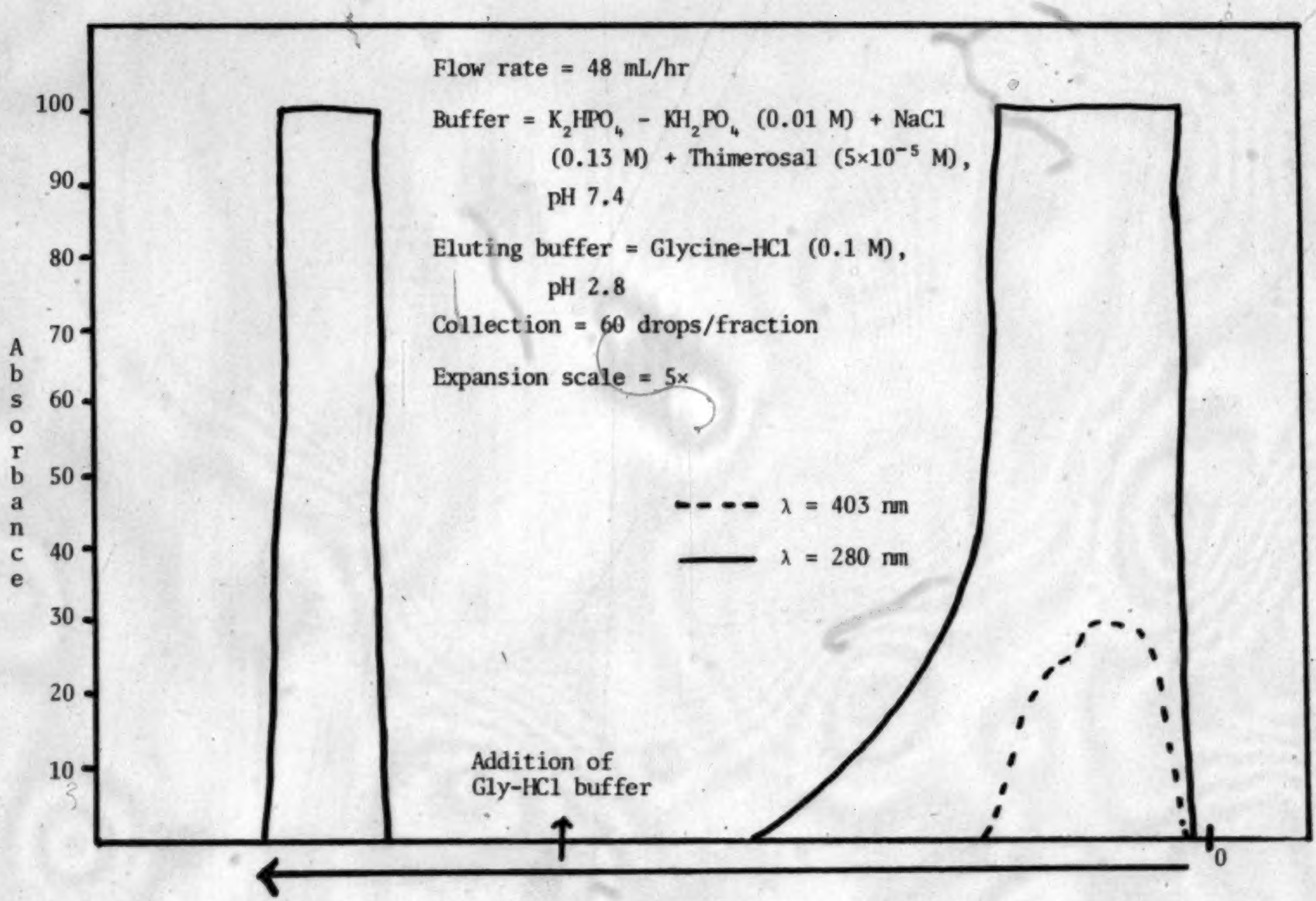
J. CONJUGATION OF HRP

Two methods of periodate conjugation were attempted, the only difference between the two being the blocking agent for the amino groups on the periodate molecule. Dinitrofluorobenzene (DNFB) does a more complete job of blocking these groups than does paraformaldehyde.

1. Paraformaldehyde

The p-formaldehyde procedure first tested was confidential information obtained by Dr. Morgante from Dr. Nakane during a two week visit to his laboratory. For this reason, the procedure will not be described and any interested readers should contact Dr. Nakane directly for details of the method [123].

Figure 10(a) illustrates the one large peak absorbing at 280 nm



Flow rate = 48 mL/hr

Buffer = K_2HPO_4 - KH_2PO_4 (0.01 M) + NaCl (0.13 M) + Thimerosal (5×10^{-5} M), pH 7.4

Eluting buffer = Glycine-HCl (0.1 M), pH 2.8

Collection = 60 drops/fraction

Expansion scale = 5×

Fig. 9 Rabbit anti-mouse IgG passaged through Protein A-Sepharose.

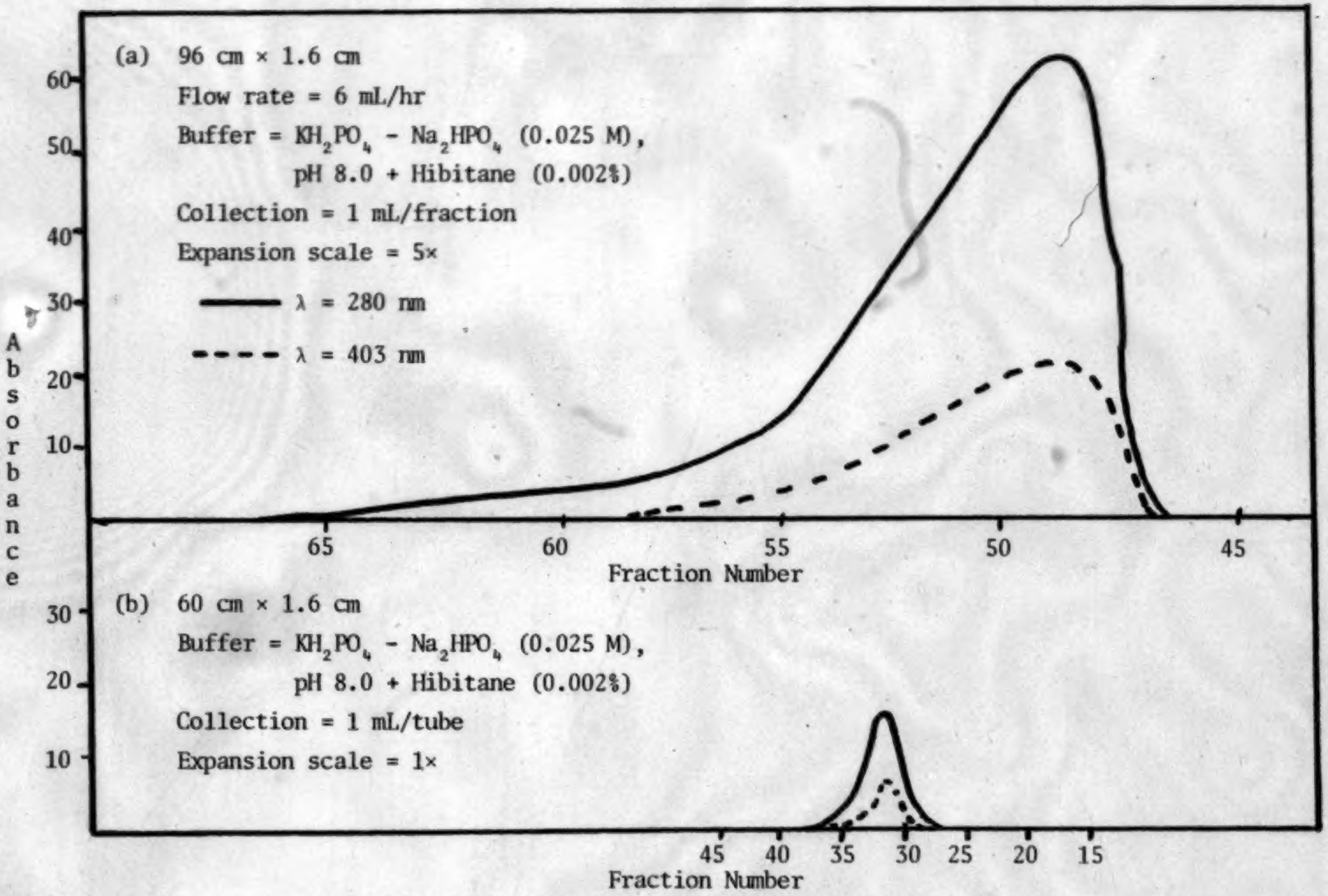


Fig. 10 (a) Rabbit anti-mouse IgG-HRP conjugate passed through Sephadex G-100.
(b) Rabbit anti-mouse IgG-HRP conjugate passed through Sephadex G-100 and Sephacryl-300.

and 403 nm after passage through a Sephadex G-100 column. As one can see, there is absolutely no separation and the entire conjugate was recovered in the void volume. The best staining results are obtained, according to Nakane and Kawaoi [3], when 2-3 moles of HRP are bound to 1 mole IgG. These conjugates have an R.Z. value of 0.6. The R.Z. values of mouse anti-WEE IgG-HRP conjugates obtained using this procedure ranged from 0.18 to 0.53--too low to expect good staining properties.

2. Dinitrofluorobenzene

The paper published by Nakane and Kawaoi [3] in 1974 describes the procedure used for the second method of periodate conjugation. Dr. K. Walls et al. [124] stresses that an E/P ratio of 1:1 (i.e., 10 mg HRP + 10 mg IgG) results in superior conjugates; therefore we decided to modify the procedure to achieve this ratio. The higher E/P ratio employed dictates the need for a higher concentration of sodium periodate ($NaIO_4$), but although Walls employs 0.08 M $NaIO_4$, we decided to use 0.06 M $NaIO_4$ to avoid the deleterious effects of higher periodate concentrations on HRP activity [3,86]. For this attempt, rabbit anti-mouse IgG was used because it had been purified by the protein A-sepharose method, whereas the mouse immunoglobulin had only been precipitated with 50% ammonium sulfate.

Ten mg of HRP were dissolved in 2 mL of 0.3 M sodium bicarbonate buffer, pH 8.1. Two-tenths of a millilitre of 1% DNFB (Eastman) in absolute ethanol was added and the solution was mixed for 1 hr at room temperature. Two mL of 0.16 M ethylene glycol were added and the solution was stirred for 1 hr at room temperature. The activated HRP was dialized against 0.01 M carbonate buffer, pH 9.5. Ten mg of rabbit anti-mouse IgG in 2 mL of 0.3 M sodium bicarbonate, pH 9.5, were added to the dialysate. After stirring for 3 hr at room temperature, 10 mg of sodium borohydride were added. The solution was left for 4 hr at 4°C without stirring. Dialysis was then carried out in 0.005 M phosphate buffer, pH 8.0.

K. PURIFICATION OF PERIODATE CONJUGATES

1. Comparison of Sephacryl-300 and Ultrogel AcA-44

A control check on the ability of Sephacryl-300 and Ultrogel AcA-44 to separate HRP and IgG was made by layering 5 mg of HRP plus 5 mg normal rabbit IgG in KH_2PO_4-Na_2HPO_4 (0.025 M), pH 8.0 + hibitane (0.002%) on each column. The total volume of each sample was about 2 mL and the conditions for running the columns were identical. Figures 11(a) and 11(b) indicate that both gels are equally useful in separating HRP from IgG. A more complete separation was achieved using Ultrogel AcA-44; however, the sample filtered through the Sephacryl-300 more quickly.

2. Comparison of Sephadex G-100 and Sephacryl-300

Both the mouse anti-WEE IgG-HRP and the rabbit anti-mouse IgG-HRP conjugates were chromatographed in a 96 cm × 1.6 cm Sephadex G-100 column and no separation occurred (Fig. 10(a)). Subsequently both conjugates were passed through a Sephacryl-300 superfine (60 cm × 1.6 cm) column to ascertain if any separation could be achieved (Fig. 10(b)). Although only the rabbit anti-mouse IgG-HRP conjugate profile is illustrated, the mouse anti-WEE IgG-HRP conjugate gave a similar profile. The peak height of the conjugate when passed through Sephacryl-300 is smaller than the peak after passage through Sephadex-100 because the expansion scale was only 1× rather than 5×.

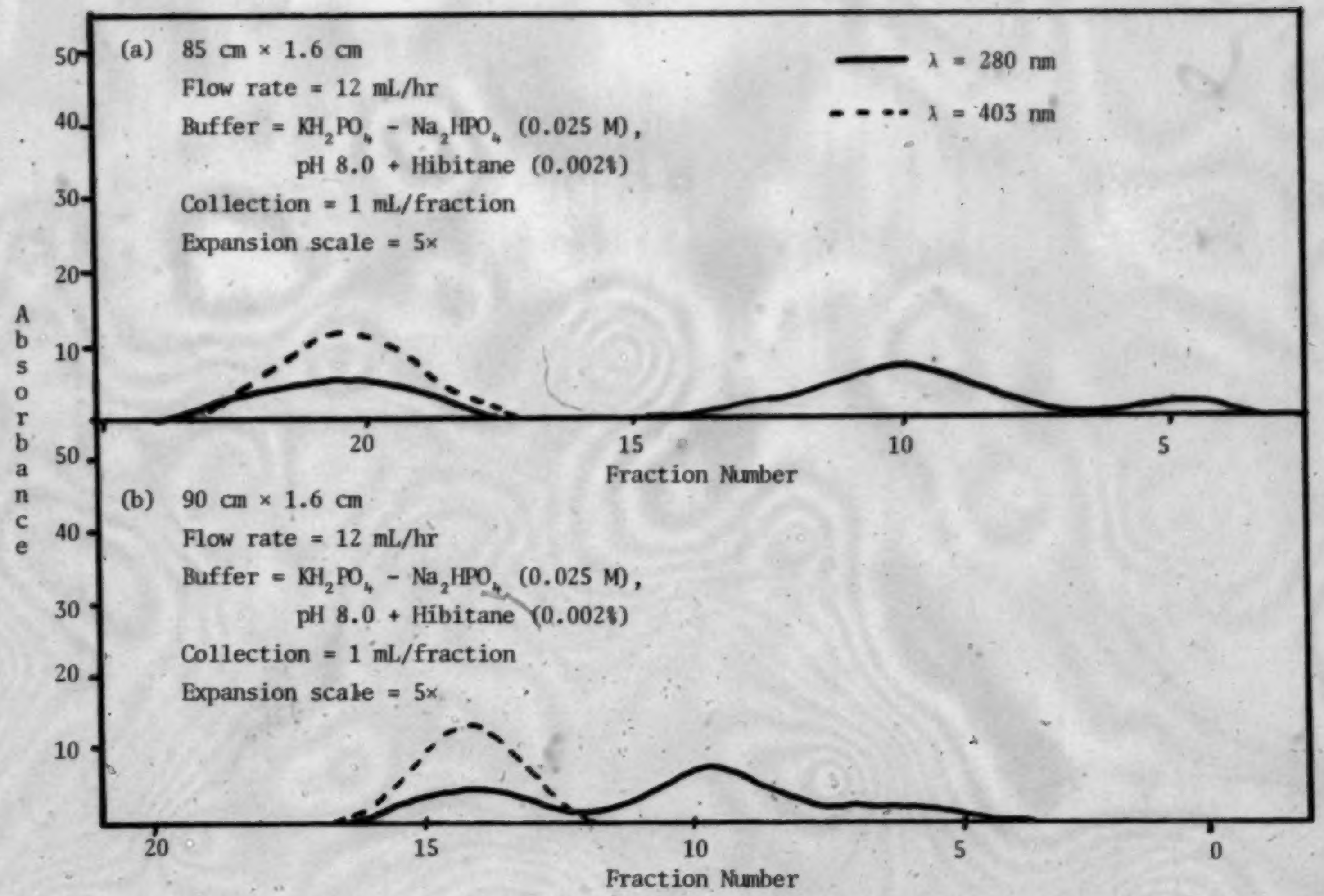


Fig. 11 *(a) Normal rabbit IgG + HRP passaged through Ultrogel AcA-44.*
(b) Normal rabbit IgG + HRP passaged through Sephacryl S-300.

L. ELISA

In spite of an R.Z. value of less than 0.6 as required by Nakane [3] and a measurable protein of less than 0.6 mg/mL, an attempt to perform a direct ELISA test was made using the mouse anti-WEE IgG-HRP conjugate since a high potency WEE virus antigen was available. Normal HeLa cells served as a control.

1. Coating of Plates

Purified WEE virus antigen and control HeLa cells were diluted 1:5, 1:10, 1:50, 1:100 and 1:1000 in coating buffer, pH 9.6 (see appendix). Two hundred microlitres of each dilution were added to each of six wells on a substrate plate (Dynatech, polysterene, #1-223-24). The plate was covered and left at 4°C overnight. Unadsorbed antigen was removed the next day by a water vacuum pump and each well was rinsed 3× by adding 0.3 mL PBS, pH 7.2 + 0.05% Tween-20 and allowing to stand for 5 min before aspirating the rinse off. Three hundred microlitres of 5% bovine albumin (BA) (Sigma) in PBS, pH 7.2, were added to each well followed by incubation for 4 hr at room temperature. The BA was then aspirated off and the wells were rinsed 3× as before. The plate was covered after the last aspiration and was kept at 4°C until needed (should be good for up to 4 weeks) [77].

2. Conjugate Dilutions and Color Development

All conjugate dilutions were made in PBS, pH 7.2 + 1% BA + 0.15% Tween-20. The mouse anti-WEE IgG-HRP conjugate was diluted 1:2, 1:4 and 1:8. Fifty microlitres of each conjugate dilution were added to each of 2 wells of the antigen and normal cell control concentration. The plate was then incubated for 2 hr at 37°C. Each well was washed 3×

as per usual.

Orthophenylenediamine was used as the chromogen for development because of its high sensitivity and low mutagenic properties [87,125].

Two hundred microlitres of orthophenylenediamine (OPDA, Sigma) substrate were added to each well. After 30 min at room temperature, the reaction was stopped by adding 25 μL of 4 N H_2SO_4 to each well. The reaction product will remain stable for 48 hours.

3. Results

All wells of the plate showed no color development, not even at a high antigen concentration of 1:5 and conjugate dilution of 1:2. Repeated tests with several conjugates were never successful in showing any enzymatic reactivity.

Chapter III

ALKALINE PHOSPHATASE

A. CHROMATOGRAPHY OF ALP ON SEPHACRYL S-300

Sephacryl S-300 was to be used for purification of the conjugate; therefore, the elution volume for the enzyme on such a column needed to be determined. One milligram of ALP (Sigma, Type VII) was chromatographed on a 90 cm × 1.6 cm Sephacryl S-300 superfine column equilibrated with Tris-HCl (0.05 M), pH 8.0, at 4°C. The fractionation range of the gel is 10,000 - 1.5 million daltons. At a flow rate of 44 mL/hr, the enzyme started to elute after 84 mL of buffer had passed through the column. No absorbance at 280 nm was recorded by the Uvicord III absorptiometer (LKB) and we attributed this to the extremely small amount of protein passed through the column. The elution volume was determined by measuring the enzymatic activities of the fractions and the findings were confirmed by a repeat run using a higher quantity of ALP (Fig. 13).

B. DETERMINATION OF ALP ENZYMATIC ACTIVITY

All the fractions recovered from passage of ALP through Sephacryl S-300 were subjected to enzymatic activity determinations (Fig. 12). The method employed was given to us by Dr. R. Stinson [126] and the kinetic measurements were read on his Beckman Acta C III UV-Visible spectrophotometer at a wavelength of 404 nm. The substrate consisted of 10 mM p-nitrophenyl phosphate (Sigma, #104-0) in 0.782 M

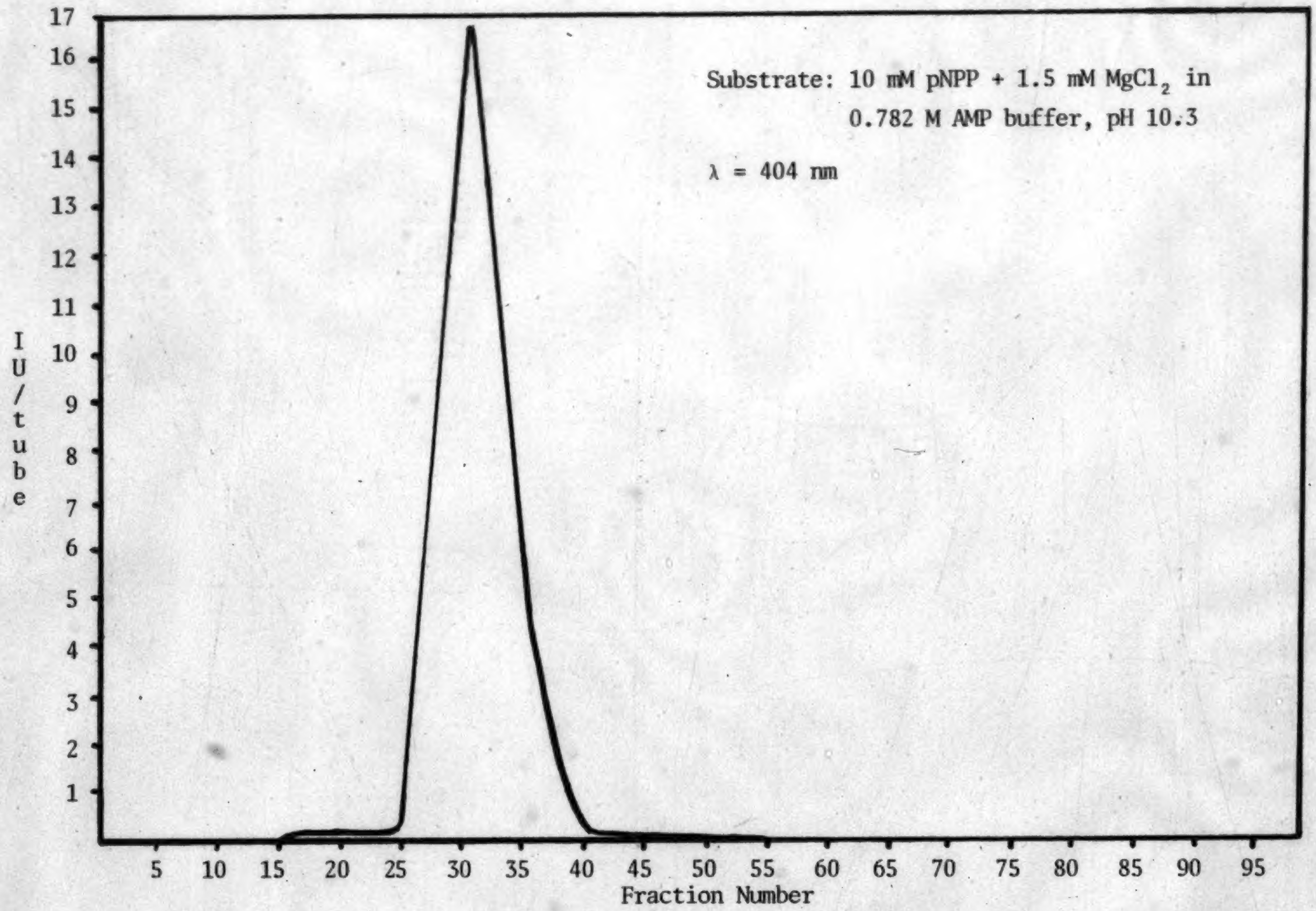


Fig. 12 Enzymatic activity determination of ALP fractions after Sephacryl S-300 chromatography.

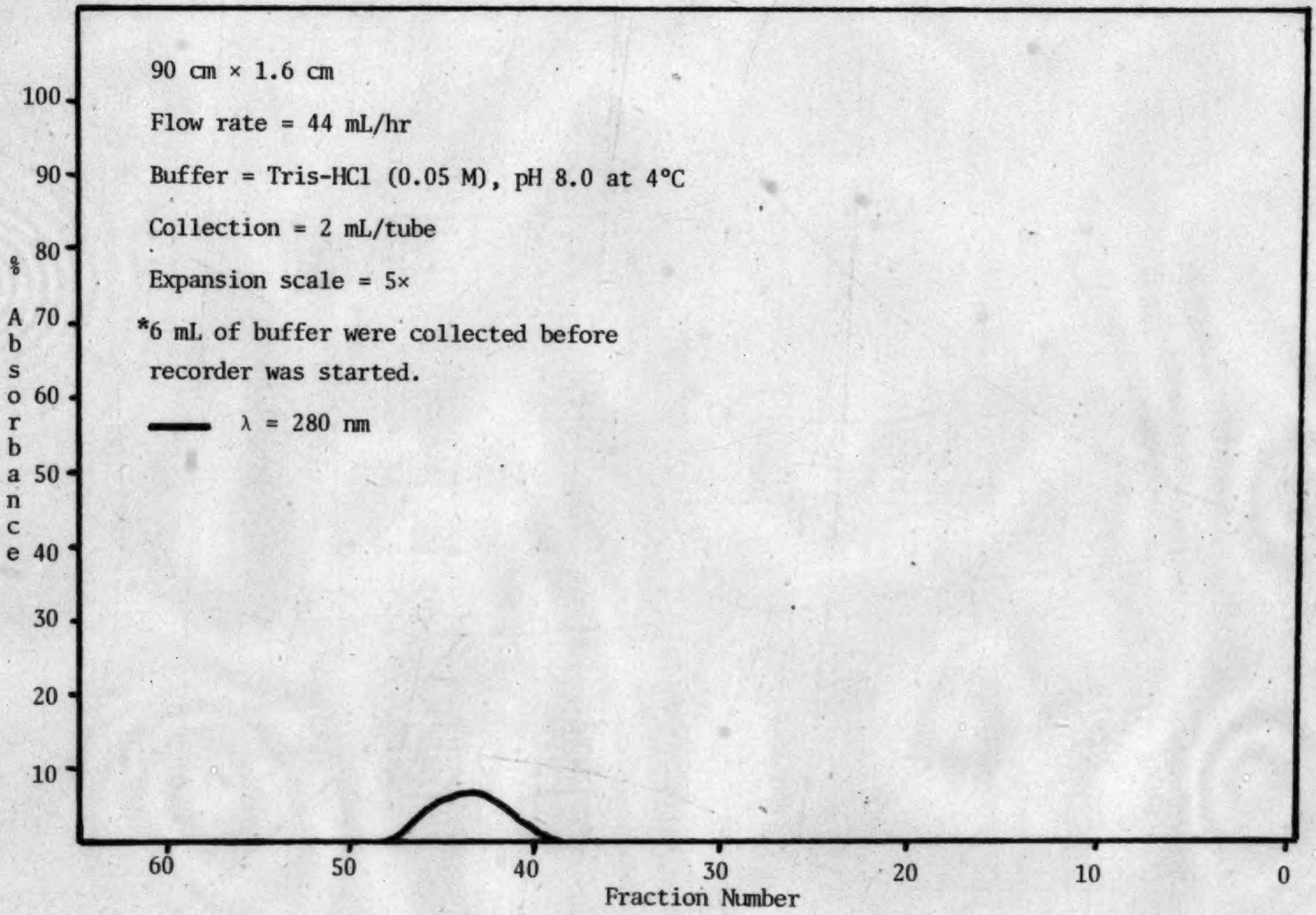


Fig. 13 ALP + rabbit IgG passaged through Sephacryl S-300.

2-amino-3-methyl-1-propanol (AMP) buffer, pH 10.3 (see appendix). One millilitre of substrate was warmed in a cuvette in a 30°C waterbath for at least 5 min. The cuvette was then placed into the spectrophotometer and the baseline was recorded. Fifty microlitres of sample were added and the mixture was mixed thoroughly. The recorder was adjusted so the enzyme kinetics showed a straight line at about a 45° angle. The enzymatic activity of each fraction was determined using the following formula:

$$\text{Enzymatic Activity} = \frac{\Delta\text{Abs/min}}{\text{extinction coefficient}} \times \frac{\text{total volume}}{\text{sample volume}} \times \begin{matrix}\text{volume in}\\ \text{tube}\end{matrix}$$

$$= \text{IU/tube (or IU/2mL sample).}$$

The extinction coefficient for ALP is 16.7.

C. CHROMATOGRAPHY OF IgG ON SEPHACRYL S-300

Two milligrams of anti-guinea pig IgG (Miles or Meloy) in Tris-HCl buffer, pH 8.0 were passed through the Sephacryl S-300 superfine. The conditions for running the column were the same as for chromatography of the enzyme. The protein started to elute after 86 mL of buffer had passed through the column--very close to the elution volume of ALP. The molecular weights of ALP and IgG are 120,000 and 160,000 daltons respectively; therefore, the theoretical molecular weight of the conjugate should be approximately 280,000 daltons. One would expect the conjugate to be eluted from the column faster than either free enzyme or antibody and separation of the conjugate should be complete.

In order to confirm the elution profiles of the IgG and ALP, 0.5 mg of IgG and 1.5 mg of ALP were mixed in 1.0 mL of Tris-HCl buffer, pH 8.0 and were passed through the Sephacryl S-300 column. The elution profile is given in Fig. 13. Again, a solitary peak eluted after 84 mL of buffer had passed through the column.

D. CONJUGATION OF ALP TO IgG

1. Engvall and Perlmann: One-step glutaraldehyde

The method of conjugation employed was the one-step glutaraldehyde procedure as first described by Avrameas [85] and later modified by Engvall and Perlmann [127]. Conjugates prepared by this method will be referred to as "conjugate #1" throughout the remainder of the text.

Three hundred microlitres of a 5 mg/mL suspension of ALP in 2.6 M $(NH_4)_2SO_4$ (Sigma, Type VII) were spun at 3000 × g for 10 min in a Servall RC-2 (SS-34 rotor) at 4°C. Two hundred microlitres of supernatant were removed and discarded. The pellet was resuspended in the remaining 0.1 mL and 0.5 mg of IgG in 0.1 mL of PBS, pH 7.2 were added to the enzyme suspension (E/P ratio = 3:1). The enzyme/antibody mixture was dialized against 4-100 mL volumes of PBS, pH 7.2 over a period of 24 hr at 4°C. After dialysis, 70% glutaraldehyde (Ingram-Bell) was diluted to 4.2% in deionized water and 10 μL were added to the enzyme/antibody mixture yielding a final concentration of 0.2% glutaraldehyde. The mixture was left at room temperature for 2½ hr after which the conjugate was dialized against Tris-HCl buffer, pH 8.0 overnight at 4°C.

2. Voller et al.: One-step glutaraldehyde

The one-step glutaraldehyde method of conjugation used by Voller and collaborators [20] was also tried since larger amounts of both enzyme and antibody were used resulting in larger quantities of conjugate than obtained using the method of Engvall and Perlmann [127], thereby reducing the number of conjugates to be prepared to complete the study.

Five milligrams of ALP (Sigma, Type VII) were centrifuged at 3000 × g as before for 15 min at 4°C. The supernatant was discarded and 2 mg of IgG in 1.0 mL of PBS, pH 7.2 were added. The ALP/IgG mixture was dialized against 4-500 mL changes of PBS, pH 7.2 overnight (E/P ratio = 2.5:1). Glutaraldehyde (Ingram-Bell, 70%) was added to a final concentration of 0.2% and the mixture was left at room temperature for 3 hr. The conjugate was dialized against 4-500 mL volumes of Tris-HCl buffer, pH 8.0 overnight. These conjugates will be referred to as "conjugate #2".

E. PURIFICATION AND ENZYMATIC ACTIVITY DETERMINATIONS OF ALP/IgG CONJUGATES

1. Conjugate #1

Conjugate #1 was chromatographed on a Sephacryl S-300 column (90 cm × 1.6 cm) to separate free enzyme and antibody from the conjugate. As indicated in Fig. 14, only one peak absorbing at 280 nm was evident after 54 mL of Tris-HCl buffer, pH 8.0 had passed through the column. No subsequent peaks were seen; however, the backside of the peak showed a moderate degree of trailing. We assumed that virtually all the ALP and IgG were conjugated and, as was expected, the conjugate came off the column in a volume less than that of either free ALP or IgG. All conjugates prepared by this method (conjugate #1) consistently eluted from the Sephacryl S-300 column after 54 mL of buffer had passed through the column.

The enzymatic activity of each fraction was determined as previously described (p. 45) and those fractions demonstrating the

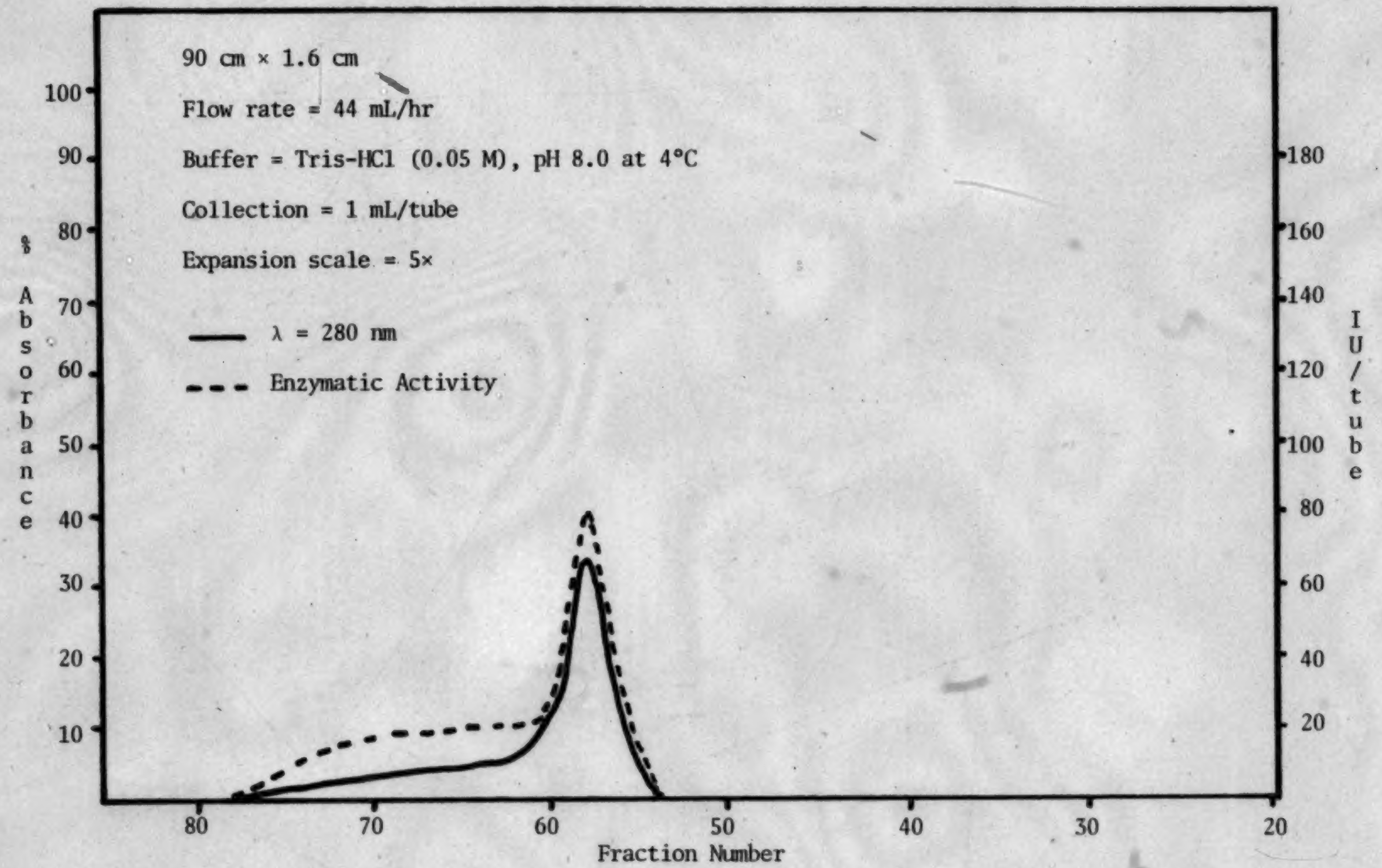


Fig. 14 Conjugate #1: passage through Sephacryl S-300 and enzymatic activity determinations.

highest enzymatic activity and the highest absorbancy at 280 nm were pooled and concentrated to 1.0 mL in an Amicon filtration cell (PM-10 membrane). The enzymatic activities of the pooled conjugates ranged from 195-245 IU/mL (Fig. 14).

2. Conjugate #2

Conjugate #2 was chromatographed on the same Sephacryl S-300 column used for conjugate #1. A very large, single peak absorbing at 280 nm was eluted after 54 mL of buffer had passed through the column (Fig. 15). The enzymatic activity of each fraction was determined and those functions containing high enzymatic activity and absorbance at 280 nm were pooled. The conjugate was concentrated to 4 mL using an Amicon filtration cell (PM-10 membrane). The enzymatic activity of these conjugates ranged from 225-255 IU/mL.

F. DETERMINATION OF CONJUGATE TITRE

1. Coating Procedure

All the buffers and solutions used in the following tests were sterilized by filtration through millipore filters (0.22 μm porosity).

The conjugates were tested against 1, 10 and 100 μg/mL concentrations of normal guinea pig (GP) IgG. The IgG dilutions were made in coating buffer (carbonate buffer, 0.07 M, pH 9.6) and 0.2 mL was added to the appropriate wells of a polysterene microtitre plate (Dynatech, U-substrate plates, #1-223-24). The plates were covered and placed overnight at 4°C. Excess antibody was removed using a vacuum pump and each well was washed 3× by adding 0.3 mL of PBS, pH 7.2 + 0.05% Tween-20, leaving for 5 min and aspirating off the rinse. Three hundred microlitres of 5% bovine albumin (Sigma) in PBS, pH 7.2 were then added

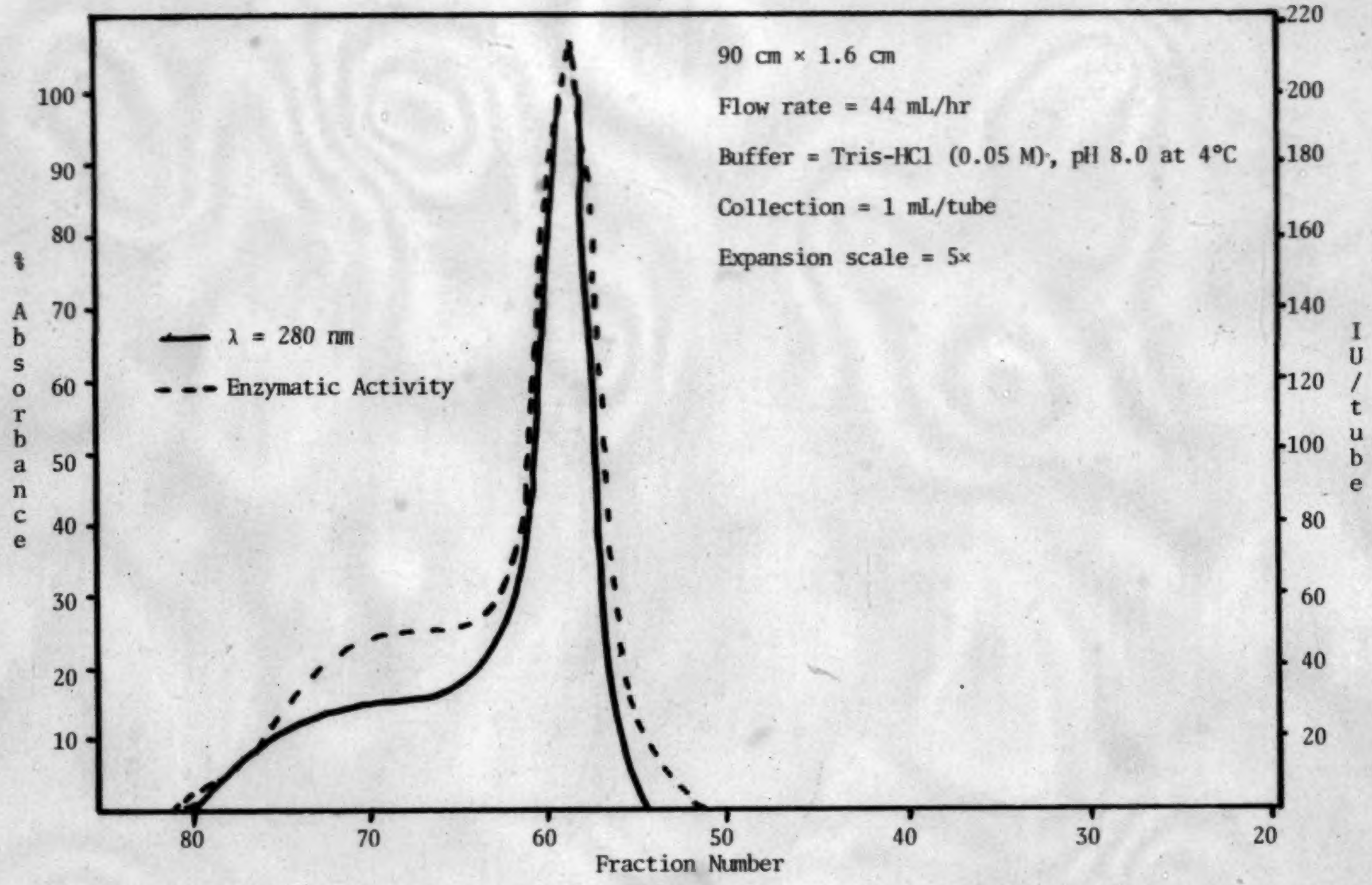


Fig. 15 Conjugate #2: passage through Sephacryl S-300 and enzymatic activity determinations.

to each well and the plates were left at room temperature for 4 hr. Each well was washed 3× as already described and the plates were kept covered at 4°C until used.

Control and blank wells were coated with carbonate buffer only and were subsequently processed identically to the other wells.

2. Test Procedure

Conjugate dilutions of 1:20, 1:100, 1:500 and 1:1000 were made in PBS, pH 7.2 + 1% bovine albumin + 0.15% Tween-20. Two hundred microlitres of the conjugate dilutions or PBS diluent were added to the appropriate wells. The plate was incubated at 37°C for 2 hr. Excess conjugate was removed and each well was washed 3× as per usual.

An amino alcohol buffer was used for the substrate as these buffers are better for this purpose than non-amino alcohol buffers. We used a 2-amino-3-methyl-1-propanol (AMP, Sigma) buffer rather than the diethanolamine (DEA) buffer used by Voller and workers [20] since AMP is a more effective buffer at the higher pH optimum required by mammalian alkaline phosphatases (pH optimum achieved with AMP is 10.3 versus 10.0 achieved using DEA). In addition, DEA may contain impurities which interfere with ALP activity [71]. The substrate employed was p-nitrophenyl phosphate (pNPP) as it gives 99.9% full color production at pH 10.5 which is much higher than that achieved by other substrates [71]. The buffer was prepared as described in the appendix.

Conjugate #1 was tested against two substrates, the first containing 1 mg/mL pNPP (Sigma, tablets, #104) used by Voller et al. [20] and the second containing 3.71 mg/mL pNPP (Sigma, phosphatase substrate, #104-0) suggested by Stinson [126]. Two hundred microlitres of substrate were added to each well and the plate was incubated for 30 min at room temperature. The reaction was stopped by adding 50 μL of 3.0 M NaOH to each well. The absorbances of the wells were read in a Flow Titretek Multiscan photometer at 405 nm.

3. Results

a. Conjugate #1

Table 3 illustrates a representative sample of numerous titrations using conjugate #1 and two different pNPP substrate concentrations (1.0 mg/mL and 3.71 mg/mL). The absorbance values observed with the 3.71 mg/mL pNPP substrate were much higher; almost twice as much absorbance in most instances than the 1 mg/mL pNPP substrate, as would be expected. The degree of non-specific adsorption of the conjugate to the plastic was monitored in the wells coated only with carbonate buffer. The effect on the absorbance by this factor was minimal when using the 1 mg/mL pNPP substrate but was emphasized when the higher substrate concentration was utilized. Wells coated with GP IgG but to which no substrate was added showed no absorbance; therefore, the absorbances in the test wells were attributed entirely to the reaction of the conjugate with the substrate. Originally, no substrate was added to the blank in order that the degree of non-specific adsorption of the conjugate to the plastic could be determined. Subsequently, substrate was added to all blanks which were treated in the same manner as the test wells.

Voller and collaborators [20] defined the optimal working concentration of conjugate as that dilution giving a 1.0 absorbance value using a 100 ng/mL IgG for coating the wells. For our IgG, we chose the 10 μg/mL IgG solution to detect the working dilution as any lower IgG concentration resulted in absorbance values of 1.0 only at the lowest

Table 3. Conjugate titre determination of conjugate #1--Engvall and Perlmann.

GP IgG in µg/mL		Conjugate Dilution 1:											
		20		100		500		1000		GP IgG		Blank	
1	*	>2.000	>2.000	0.084	0.463	0.098	0.100	0.051	0.045	9.997	0.024	0.000	0.003
	+	>2.000	>2.000	0.934	0.941	0.178	0.206	0.111	0.086	9.992	0.001	9.996	9.984
10	*	>2.000	>1.850	0.394	0.482	0.111	0.105	0.055	0.058	0.001	0.004	0.000	9.995
	+	>2.000	>2.000	1.194	1.177	0.193	0.188	0.139	0.093	9.987	9.998	0.000	9.984
100	*	>2.000	1.612	0.759	0.428	0.131	0.083	0.058	0.048	0.007	0.015	0.002	0.004
	+	>2.000	>2.000	0.909	0.866	0.224	0.201	0.081	0.123	9.978	9.994	9.997	9.991
Coating	*	0.062	0.059	0.048	0.064	0.060	0.059	0.058	0.058				
Buffer	+	0.226	0.254	0.234	0.228	0.199	0.184	0.209	0.192				

* Substrate consisted of 1 mg/mL pNPP (Sigma, #104).

+ Substrate consisted of 3.71 mg/mL pNPP (Sigma, #104-0).

Conjugate was added to all wells coated with coating buffer.

Duplicate readings under each conjugate dilution represent the readings from two test wells.

dilutions of conjugate, meaning that a great deal of conjugate would be needed for each test. Table 3 shows that if the more concentrated substrate was used, a conjugate dilution of 1:100 (N.B. background absorbance value must be subtracted from the test absorbance) was optimal. Further conjugate dilutions between 1:20 and 1:100 would have had to be made to determine the optimal dilution using the less concentrated substrate.

A subsequent conjugate titration using dilutions of 1:50, 1:75, 1:100 and 1:200 in wells coated with 10 μg/mL IgG helped to pinpoint the working strength of the preparation. Table 4 illustrates that a conjugate dilution of approximately 1:150 using the pNPP substrate should have been very close to the optimal dilution (the conjugate used in Table 4 is different from the one used in Table 3).

We decided to use the 1.0 mg/mL pNPP substrate in future testing so that background absorbances would not have to be subtracted from the test values and the test could be read directly. In addition, the abovementioned substrate is less expensive to prepare.

b. Conjugate #2

Conjugate #2 was tested against the same GP IgG concentrations as were used for conjugate #1 for comparison purposes (Table 5). Again, a 10 μg/mL concentration of IgG gave almost as high a conjugate endpoint as the 100 μg/mL IgG (1:100 and 1:120 respectively). The readings obtained using 1 μg/mL IgG failed to reach an absorbance of 1.0 even at the lowest conjugate dilution (1:60). No background absorbance was seen due to non-specific adsorption of conjugate to the plastic and the IgG did not lend any background absorbance itself. The optimal working strength for this conjugate was a 1:100 dilution.

Table 4. Conjugate titre determination of conjugate #1--Engvall and Perlmann.

GP IgG		Conjugate Dilution 1:											
in µg/mL		50		75		100		200		GP IgG		Blank	
10	*	>2.000	>2.000	1.828	1.853	1.539	1.467	0.678	0.720	9.917	9.916	9.987	9.906
	+	>2.000	>2.000	>2.000	>2.000	>2.000	>2.000	1.421	1.402	0.073	0.077	9.969	0.082
Coating	*	9.854	9.857	9.859	9.855	9.867	9.846	9.856	9.850	9.847	9.841	9.883	9.847
Buffer	+	0.150	0.136	0.118	0.120	0.130	0.117	0.126	0.133	0.084	0.094	9.907	0.088

* Substrate consisted of 1 mg/mL pNPP (Sigma, #104).

+ Substrate consisted of 3.71 mg/mL pNPP (Sigma, #104-0).

Conjugate was added to all wells coated with coating buffer.

Duplicate readings under each conjugate dilution represent the readings from two test wells.

Table 5. Conjugate titre determination of conjugate #2--Voller.

GP IgG in µg/mL		Conjugate Dilution 1: 60		80		120		240		480		Blank	
1	*	0.884	0.785	0.672	0.562	0.401	0.356	0.209	0.180	0.100	0.086	0.001	9.999
	+	0.015	0.009	0.013	0.013	0.014	0.019	0.015	0.013	0.018	0.016	9.999	0.000
Coating Buffer		0.009	0.004	0.013	0.001	0.019	0.004	0.013	0.006	0.016	0.003	0.000	0.003
10	*	1.610	1.499	1.504	1.383	0.903	0.792	0.492	0.487	0.269	0.239	9.992	9.991
	+	9.995	9.995	9.999	9.989	9.991	9.996	9.989	9.987	9.995	9.994	9.988	9.994
Coating Buffer		9.988	9.967	9.983	9.977	9.987	9.970	9.986	9.966	9.983	9.968	9.988	9.968
100	*	>2.000	1.963	1.992	1.672	1.209	1.088	0.621	0.543	0.345	0.316	9.991	9.973
	+	0.014	0.006	0.009	0.009	9.990	0.012	9.990	0.015	9.993	0.011	9.986	0.001
Coating Buffer		9.969	9.993	9.990	9.970	9.988	9.965	9.992	9.965	0.012	9.968	9.981	9.967

* Coated wells with conjugate added.

+ Coated wells without conjugate added.

All wells coated with carbonate buffer had conjugate added.

Substrate was 1 mg/mL pNPP (Sigma, #104).

Duplicate readings under each conjugate dilution represent the readings from two separate wells.

c. Pooled Conjugates

The conjugates prepared by the Engvall-Perlmann [127] and Voller et al. [20] methods were pooled since both types of conjugates eluted in the same fraction after passage through Sephacryl S-300 column chromatography and the enzymatic activities were relatively similar. The conjugates were pooled to ensure that a large amount of conjugate with constant properties was available to perform all subsequent ELISA tests. The conjugate working strength was determined against 10 μg/mL IgG to be 1:200 with an enzymatic activity of roughly 225 IU/mL (Table 6). Blanks were treated in the same way as the test wells.

G. ANTIGEN TITRATIONS OF GROUP B COXSACKIEVIRUSES

1. Coating of Plates

Partially purified Coxsackievirus antigens in HeLa cells were diluted 1:100, 1:1000 and 1:10,000 in coating buffer. Each polysterene plate (Dynatech, U-substrate plates, #1-223-24) was coated as previously described (p. 52) with the following controls:

a. *Antigen control.* Wells were coated with Coxsackie B-virus-infected or normal HeLa cells to which PBS diluent was added instead of immune serum. Conjugate and substrate were added.

b. *Normal serum antigen control.* Wells coated with virus-infected and normal HeLa cells had normal GP serum added followed by conjugate and substrate.

c. *Immune serum control.* Wells coated with carbonate buffer had the two lowest dilutions of GP anti-Coxsackievirus serum added plus conjugate and substrate.

d. *Normal serum control.* Wells coated with carbonate buffer

Table 6. Conjugate titre determination of pooled conjugate #1 and conjugate #2.

GP IgG	Conjugate Dilution 1:													
in µg/mL	60		80		100		120		240		480		Blank	
10	>2.000	>2.000	>2.000	>2.000	1.896	1.657	1.563	1.450	0.857	0.795	0.432	0.379	9.997	9.989
Coating Buffer	0.007	0.006	0.004	9.996	9.995	9.997	0.020	0.017	0.034	0.010	0.000	0.001	9.995	9.997

Substrate was 1 mg/mL pNPP (Sigma, tablets, #104).

Conjugate was added to all wells coated with coating buffer.

Duplicate readings under each conjugate dilution represent the readings from two test wells.

had the two lowest dilutions of normal GP serum added plus conjugate and substrate.

e. *Normal cell control*. Uninfected HeLa cells (purified like the virus-infected cells) were diluted the same as the infected cells and GP anti-Coxsackie B-virus serum plus conjugate and substrate were added.

f. *Blank*. Wells were coated with carbonate buffer to which PBS diluent, conjugate and substrate were added.

2. Test Procedure

Dilutions of the GP antiserum corresponding to the Coxsackievirus antigen were made in PBS, pH 7.2 + 0.05% Tween-20 and were added in 0.2 mL amounts to the appropriate wells. The plates were incubated for 2 hr at 37°C after which excess antiserum was removed and each well was washed 3×. Two hundred microlitres of a 1:200 dilution of the conjugate in PBS, pH 7.2 + 1% bovine albumin + 0.15% Tween-20 was added to each well and the plates were reincubated for 2 hr at 37°C. Excess conjugate was removed followed by three washings. Two hundred microlitres of substrate (1 mg pNPP/mL) was added to each well and the plate was incubated for 30 min at 30°C. The reaction was stopped by addition of 50 µL of 3.0 M NaOH and the absorbances were measured in a Flow Titretek Multiscan photometer at 405 nm. All incubations at 37°C were performed in a moist chamber to avoid evaporation of the reagents.

3. Results

Tables 7-12 give the endpoint titres of the individual Coxsackie B-viruses. In every titration the control wells demonstrated negligible absorbance except for the normal cell controls which showed some

Table 7. Antigen titration of Coxsackievirus B1.

Antigens	Anti-Cox B1 1:							Cox B1 & HeLa + PBS	Cox B1 & HeLa + N.Serum* 1:40	Coating Buffer + Anti-B1 1:40 & 1:80	Coating Buffer + N.Serum* 1:40 & 1:80	Blank
	40	80	160	320	640	1280	2560					
Cox B1	0.764	0.481	0.541	0.384	0.256	0.199	0.172	0.001	0.071	0.065	9.938	9.998
10^{-2}	0.445	0.589	0.472	0.323	0.249	0.122	0.063	9.935	0.008	9.956	9.949	9.954
HeLa Cells	0.335	0.275	0.212	0.165	0.128	0.034	0.000	9.954	0.082	9.918	0.018	9.954
10^{-2}	0.376	0.410	0.310	0.282	0.295	0.250	0.128	0.029	9.983	0.002	0.005	0.084
Cox B1	0.517	0.510	0.401	0.332	0.338	0.209	0.231	9.936	9.966			9.931
10^{-3}	0.535	0.431	0.377	0.300	0.231	0.120	0.070	9.981	9.963			0.046
HeLa Cells	0.095	0.078	0.068	0.048	0.045	0.047	0.007	9.985	9.967			0.057
10^{-3}	0.157	0.120	0.138	0.094	0.038	0.073	0.060	0.019	9.978			0.015
Cox B1	0.598	0.391	0.326	0.339	0.196	0.188	0.113	0.054	0.073			0.061
10^{-4}	0.327	0.294	0.273	0.213	0.127	0.091	0.043	9.972	9.990			0.002
HeLa Cells	0.005	9.971	9.925	9.927	9.946	9.928	9.928	9.927	0.011			9.972
10^{-4}	0.105	0.055	0.056	0.064	0.058	0.063	0.023	0.047	9.958			9.965

* N. Serum = Normal G. Pig Serum

Table 8. Antigen titration of Coxsackievirus B2.

Antigens	Anti-Cox B2 1:							Cox B2 & HeLa + PBS	Cox B2 & HeLa + N.Serum* 1:40	Coating Buffer + Anti-B2 1:40 & 1:80	Coating Buffer + N.Serum* 1:40 & 1:80	Blank
	40	80	160	320	640	1280	2560					
Cox B2	0.576	0.662	0.539	0.468	0.317	0.180	0.124	9.925	9.993	0.099	0.005	9.998
10^{-2}	0.531	0.656	0.571	0.451	0.313	0.193	0.099	0.009	9.963	0.047	0.007	9.954
HeLa Cells	0.497	0.514	0.389	0.327	0.222	0.169	0.100	9.995	0.004	9.960	9.953	9.931
10^{-2}	0.590	0.571	0.500	0.350	0.233	0.179	0.163	9.986	0.012	0.007	9.999	9.975
Cox B2	0.342	0.331	0.303	0.272	0.205	0.181	0.115	0.019	0.004			9.986
10^{-3}	0.263	0.276	0.177	0.147	0.110	0.130	0.021	9.946	0.012			0.008
HeLa Cells	9.994	9.946	9.939	9.937	9.944	9.935	9.925	0.000				0.002
10^{-3}	0.062	0.039	0.044	0.042	0.042	0.037	0.027	9.998				9.991
Cox B2	0.119	0.102	0.140	0.127	0.090	0.067	0.017	9.937				9.997
10^{-4}	0.214	0.137	0.128	0.094	0.056	0.034	0.020	9.941				0.005
HeLa Cells	0.014	0.019	0.010	0.008	0.010	0.006	9.995	0.003				9.994
10^{-4}	0.016	0.021	0.040	0.019	0.034	0.024	0.024	0.000				9.988

* N. Serum = Normal G. Pig Serum

Table 9. Antigen titration of Coxsackievirus B3.

	Anti-Cox B3 1:							Cox B3 & HeLa + PBS	Cox B3 & HeLa + N.Serum* 1:40	Coating Buffer + Anti-B3 1:40 & 1:80	Coating Buffer + N.Serum* 1:40 & 1:80	
Antigens	40	80	160	320	640	1280	2560					Blank
Cox B3	0.291	0.037	0.184	0.206	0.090	0.076	9.990	9.948	0.002	9.951	9.980	9.980
10^{-2}	0.293	0.279	0.293	0.272	0.201	0.146	0.040	9.985	9.988	0.001	9.965	9.988
HeLa Cells	0.153	0.103	0.059	0.030	0.115	0.125	0.001	9.958	9.969	9.960	9.991	9.989
10^{-2}	0.145	0.091	0.063	0.068	0.026	0.076	0.067	9.952	9.976	9.964	9.962	9.992
Cox B3	0.186	0.172	0.066	0.151	0.131	0.108	0.125	9.995	0.004			9.956
10^{-3}	0.098	0.093	0.125	0.082	0.084	0.019	0.044	9.981	9.948			9.917
HeLa Cells	0.108	0.022	0.064	0.005	0.022	9.988	0.045	9.975	9.969			9.949
10^{-3}	9.834	0.076	0.019	0.021	0.008	9.988	0.036	0.039	9.943			9.985

* N. Serum = Normal G. Pig Serum

Table 10. Antigen titration of Coxsackievirus B4.

	Anti-Cox B4 1:							Cox B4 & HeLa + PBS	Cox B4 & HeLa + N.Serum* 1:40	Coating Buffer + Anti-B4 1:40 & 1:80	Coating Buffer + N.Serum* 1:40 & 1:80	Blank
Antigens	40	80	160	320	640	1280	2560					
Cox B4	0.168	0.213	0.158	0.187	0.172	0.232	0.139	0.028	0.032	0.048	9.975	0.030
10^{-2}	0.167	0.167	0.165	0.187	0.160	0.196	0.125	0.026	0.033	0.040	9.991	0.017
HeLa Cells	0.117	0.114	0.129	0.098	0.118	0.084	0.069	0.025	0.047	0.021	9.998	0.012
10^{-2}	0.103	0.105	0.125	0.073	0.119	0.097	0.066	0.022	0.060	0.042	0.007	0.021
Cox B4	0.206	0.279	0.378	0.239	0.276	0.254	0.180	0.029	0.043			0.024
10^{-3}	0.235	0.327	0.267	0.247	0.291	0.215	0.149	0.028	0.060			0.030
HeLa Cells	0.047	0.059	0.042	0.025	0.025	0.024	0.014	0.011	0.046			0.025
10^{-3}	0.040	0.040	0.042	0.034	0.023	0.022	0.020	0.016	0.040			0.015
Box B4	0.059	0.054	0.063	0.070	0.054	0.035	0.023	0.002				0.016
10^{-4}	0.086	0.045	0.068	0.060	0.039	0.035	0.028	0.006				0.002
HeLa Cells	0.051	0.048	0.020	0.012	0.037	9.991	0.030	0.015				0.002
10^{-4}	0.138	0.039	0.020	0.016	0.008	0.010	0.006	0.023				0.012

* N. Serum = Normal G. Pig Serum

Table 11. Antigen titration of Coxsackievirus B5.

	Anti-Cox B5 1:											
Antigens	40	80	160	320	640	1280	2560	Cox B5 & HeLa + PBS	Cox B5 & HeLa + N.Serum* 1:40	Coating Buffer + Anti-B5 1:40 & 1:80	Coating Buffer + N.Serum* 1:40 & 1:80	Blank
Cox B5	0.303	0.246	0.177	0.097	0.146	0.029	0.050	0.004	0.008	0.057	0.021	0.013
10^{-2}	0.266	0.221	0.180	0.141	0.101	0.083	0.069	0.014	0.012	0.030	0.014	0.018
HeLa Cells	0.359	0.281	0.260	0.238	0.177	0.119	0.092	9.995	0.021	0.022	0.003	0.023
10^{-2}	0.377	0.314	0.291	0.198	0.152	0.112	0.087	0.002	0.014	0.006	9.995	0.017
Cox B5	0.317	0.248	0.224	0.147	0.118	0.094	0.061	0.002	0.010			9.992
10^{-3}	0.314	0.272	0.222	0.172	0.126	0.094	0.073	0.014	0.015			9.986
HeLa Cells	0.088	0.076	0.065	0.037	0.029	0.020	0.018	0.015	0.003			9.990
10^{-3}	0.071	0.064	0.051	0.029	0.025	0.012	0.008	0.004	9.998			9.994
Cox B5	0.168	0.127	0.118	0.093	0.069	0.045	0.022	9.993	9.998			0.005
10^{-4}	0.146	0.129	0.109	0.079	0.047	0.033	0.013	9.989	9.986			0.007
HeLa Cells	0.041	0.020	0.013	0.002	9.999	9.998	9.997	9.985	9.995			0.012
10^{-4}	0.033	0.019	0.008	0.003	9.998	9.997	9.991	9.983	9.994			9.999

* N. Serum = Normal G. Pig Serum

Table 12. Antigen titration of Coxsackievirus B6.

	Anti-Cox B6 1:							Cox B6 & HeLa + PBS	Cox B6 & HeLa + N.Serum* 1:40	Coating Buffer + Anti-B6 1:40 & 1:80	Coating Buffer + N.Serum* 1:40 & 1:80	
Antigens	40	80	160	320	640	1280	2560					Blank
Cox B6	0.477	0.313	0.162	0.116	0.085	0.067	0.052	0.028	0.032	0.048	0.003	0.030
10^{-2}	0.332	0.231	0.126	0.088	0.057	0.013	0.008	0.026	0.033	0.040	9.999	0.017
HeLa Cells	0.269	0.141	0.127	0.073	0.045	0.025	0.031	0.025	0.047	0.021	0.008	0.012
10^{-2}	0.237	0.125	0.107	0.075	0.045	0.045	0.019	0.022	0.060	0.042	9.991	0.021
Cox B6	0.333	0.161	0.152	0.130	0.058	0.072	0.049	0.029	0.043			0.034
10^{-3}	0.394	0.276	0.130	0.098	0.013	0.072	0.035	0.028	0.060			0.030
HeLa Cells	0.030	0.072	0.019	0.013	0.021	0.004	9.986	0.011	0.046			0.025
10^{-3}	0.090	0.044	0.025	0.015	0.030	0.019	0.001	0.016	0.040			0.015
Cox B6	0.258	0.089	0.185	0.098	0.062	0.038	0.020	0.002				0.016
10^{-4}	0.213	0.161	0.147	0.108	0.054	0.032	0.009	0.006				0.002
HeLa Cells	0.124	0.071	0.085	0.072	0.054	0.048	0.041	0.015				0.002
10^{-4}	0.128	0.059	0.041	0.119	0.048	0.040	0.041	0.023				0.012

* N. Serum = Normal G. Pig Serum

absorbance at the highest concentrations of antigen and antisera. The effect of the background absorbance was rectified when higher dilutions of the normal and virus-infected HeLa cells were used to coat the plates. Table 13 gives a summary of the optimal antigen concentrations to be used for cross-reactivity testing of the antisera. The endpoint antigen titre was taken as that concentration giving an absorbance of 0.150 or more (after the background absorbance of the corresponding normal cell control was subtracted) at the highest dilution of homotypic antiserum.

Table 13. Summary of optimal Coxsackievirus antigens and antisera dilutions.

	Optimal	
Virus	Antigen Dilution	Homotypic Antiserum Dilution
Cox B1	10^{-3}	1:640
Cox B2	10^{-3}	1:320
Cox B3	10^{-2}	1:320
Cox B4	10^{-3}	1:1280
Cox B5	10^{-3}	1:160
Cox B6	10^{-3}	1:80

H. DETECTION OF NON-SPECIFICITY OF MICROTITRE PLATES

Since our immune sera were obtained by inoculating GP with viral antigen prepared in HeLa cells, we wanted to establish the cross-reactivity of the Coxsackie B-viruses employing antigens prepared in

AGMK cells to ensure the absence of heterotypic reactions which could have arisen due to anti-HeLa antibodies. Checkerboard titrations were performed with each new Coxsackie B-AGMK antigen in exactly the same manner by which the Coxsackie B-HeLa antigens were previously titrated (see Tables 7-12). With great dismay, we soon found that the controls to which normal or immune GP sera were added all displayed absorbance readings as high as those obtained in the test (Table 14). These titrations were repeated many times since we were at a loss as to how to explain the sudden appearance of such gross non-specific findings. The addition of 1, 2 and 5% fetal calf serum to the buffer diluents did not reduce the non-specificity of our findings.

Suddenly it occurred to us that the only new variable introduced into our system was a new lot of polysterene plates (Dynatech, U-substrate plates, #1-223-24). In order to assess our suspicions a series of experiments was set up in which no viral antigen or antisera were employed. Only sera obtained from non-immunized guinea pigs were used to test the following four microtitre plates:

1. polyvinyl, flat bottom (Dynatech, flexvinyl, #1-220-29)
2. polyvinyl, round bottom (Dynatech, flexvinyl, #1-220-24)
3. polysterene, flat bottom (Limbro)
4. polysterene, U-substrate (Dynatech, Immulon, #1-223-24).

1. Test Procedure

Each well was coated with carbonate buffer only. After three washes, doubling dilutions of normal GP serum from 1:20 - 1:640 made in either PBS or in PBS + 1% fetal calf serum (FCS) were added in 0.2 mL amounts. Two sets of blanks were run, one with PBS added, the other

Table 14. Example of nonspecific reaction in Coxsackievirus B2 antigen titration.

Antigens	Anti-Cox B2 1:							Cox B2 & AGMK + PBS	Cox B2 10^{-1} + N.Serum* 1:20 & 1:40	Coating Buffer + Anti-B2 1:20 & 1:40	Coating Buffer + N.Serum* 1:20 & 1:40	Blank
	20	40	80	160	320	640	1280					
Cox B2	1.481	1.562	1.496	1.363	1.108	1.184	0.698	0.010	1.650	>2	1.641	0.011
10^{-1}	0.468	1.554	1.615	1.389	1.211	0.892	0.616	0.015	1.626	>2	1.660	9.986
AGMK Cells	1.494	1.576	1.667	1.290	1.276	1.158	0.658	0.016	1.650	>2	1.405	0.018
10^{-1}	1.541	1.576	1.574	1.220	1.194	1.024	0.646	0.025	1.644	>2	1.421	0.007
Cox B2	1.546	1.513	1.487	1.146	1.244	1.123	0.605	0.020				9.998
10^{-2}	1.472	1.578	1.549	1.346	1.285	1.101	0.664	0.002				9.991
AGMK Cells	1.522	1.587	1.486	1.445	1.482	1.141	0.647	0.033				0.014
10^{-2}	1.470	1.640	1.467	1.442	1.589	0.893	0.614	0.003				9.990

* N. Serum = Normal G. Pig Serum

Table 14 (cont'd)

Antigens	Anti-Cox B2 1:							Cox B2 & AGMK + PBS	Cox B2 10^{-3} + N.Serum* 1:20 & 1:40	Coating Buffer + Anti-B2 1:20 & 1:40	Coating Buffer + N.Serum* 1:20 & 1:40	Blank
	20	40	80	160	320	640	1280					
Cox B2	1.134	1.281	1.183	1.038	1.148	0.734	0.410	0.001	1.037	1.299	1.017	0.028
10^{-3}	1.118	1.195	1.205	1.096	0.963	0.831	0.471	9.988	1.046	0.938	1.067	9.993
AGMK Cells	1.023	1.127	1.174	0.995	1.001	0.792	0.456	9.976	0.773	1.141	0.996	0.019
10^{-3}	1.081	1.184	1.276	1.109	0.889	0.649	0.460	0.025	0.964	1.200	0.945	0.015
Cox B2	1.054	1.124	1.071	1.014	0.959	0.726	0.469	9.989				9.993
10^{-4}	1.123	1.240	1.072	0.959	0.934	0.683	0.403	9.980				0.004
AGMK Cells	0.994	1.331	1.204	1.254	1.063	0.907	0.471	0.025				0.013
10^{-4}	1.071	1.103	1.290	1.149	1.150	0.783	0.335	9.995				9.980

* N. Serum = Normal G. Pig Serum

with PBS + 1% FCS. After incubation for 2 hr at 37°C, the wells were washed 3× and 0.2 mL of conjugate (1:200) were added. The absorbances were read in the Flow Titretek Multiscan photometer at 405 nm.

2. Results

The polyvinyl flat bottom plates demonstrated an equally high degree of normal GP serum adsorption whether the serum diluent was PBS or PBS + 1% FCS, even at a serum dilution of 1:640 (Table 15). The polyvinyl round bottom plate did not fit into the photometer; however, visually, all the wells including the blanks were yellow in color. Polysterene flat bottom plates also showed a high degree of adsorption which was uncorrected by addition of 1% FCS (Table 16), but the extent of non-specific adsorption was not as high as that seen with the polyvinyl plates. The polysterene U-substrate plates showed the lowest degree of non-specific adsorption of the GP serum although addition of 1% FCS to the diluent did not reduce the amount bound (Table 17). In all plates except the polyvinyl round bottom plate, the blanks had zero absorbances proving that the conjugate did not bind non-specifically.

Many further tests were needed to thoroughly define the parameters of non-specific protein binding to microtitre plates and to arrive at a solution for circumventing the problem. The research for this thesis ended on this note due to lack of time and the cross-reactivity of the Group B Coxsackieviruses was not determined. Dr. Morgante is continuing the investigation of the plastics at the present time and will complete the cross-reactivity testing when the problem has been overcome.

Table 15. Nonspecific adsorption in polyvinyl, flat bottom plates (Dynatech, flexvinyl, #1-220-29).

Normal GP Serum Diluent	Normal G. Pig Serum 1:						Carb. Buffer + 1% FCS + PBS	Carb. Buffer + PBS
	20	40	80	160	320	640		
PBS	0.618	0.574	0.327	0.326	0.144	0.125	9.936	9.984
	0.516	0.452	0.214	0.215	0.032	0.013	0.068	9.930
PBS	0.771	0.693	0.548	0.341	0.204	0.183	0.055	0.087
	0.544	0.483	0.343	0.130	9.991	9.967	0.131	0.067
PBS	0.607	0.883	0.618	0.334	0.228	0.190	9.994	0.059
	0.458	0.739	0.452	0.205	0.079	0.043	0.102	0.008
1% FCS	0.530	0.460	0.404	0.260	0.229	0.166	9.993	0.094
+ PBS	0.424	0.368	0.318	0.163	0.128	0.083	0.155	0.007
1% FCS	0.633	0.633	0.541	0.305	0.286	0.259	0.000	0.032
+ PBS	0.550	0.581	0.464	0.238	0.213	0.193	0.162	9.952
1% FCS	0.582	0.673	0.477	0.260	0.315	0.180	0.005	0.112
+ PBS	0.423	0.533	0.327	0.118	0.173	0.033	0.194	0.085

PBS = Phosphate Buffered Saline

FCS = Fetal Calf Serum

Table 16. Nonspecific adsorption in polysterene, flat bottom plates (Limbro).

Normal GP Serum Diluent	Normal G. Pig Serum 1:						Carb. Buffer + 1% FCS + PBS	Carb. Buffer + PBS
	20	40	80	160	320	640		
PBS	0.487	0.322	0.246	0.143	0.116	0.050	9.988	0.033
	0.448	0.279	0.206	0.101	0.069	0.008	9.988	0.034
PBS	0.390	0.370	0.211	0.113	0.086	0.039	0.007	0.020
	0.396	0.375	0.219	0.117	0.089	0.043	0.010	0.021
PBS	0.436	0.332	0.179	0.087	0.076	0.035	0.038	9.995
	0.477	0.375	0.217	0.125	0.113	0.072	0.022	9.993
1% FCS + PBS	0.423	0.254	0.198	0.106	0.056	0.025	0.010	0.010
	0.433	0.259	0.209	0.112	0.063	0.029	0.000	0.029
1% FCS + PBS	0.429	0.264	0.234	0.128	0.045	0.015	0.002	0.011
	0.440	0.298	0.200	0.130	0.049	0.026	9.993	9.988
1% FCS + PBS	0.440	0.276	0.250	0.141	0.054	0.024	9.986	0.004
	0.440	0.300	0.200	0.128	0.051	0.021	0.028	9.985

PBS = Phosphate Buffered Saline

FCS = Fetal Calf Serum

. Table 17. Nonspecific Adsorption in polysterene, U-substrate plates (Dynatech, Immulon, #1-223-24).

Normal GP Serum Diluent	Normal G. Pig Serum 1:						Carb. Buffer + 1% FCS + PBS	Carb. Buffer + PBS
	20	40	80	160	320	640		
PBS	0.151	0.176	0.093	0.053	0.019	0.006	9.994	9.994
	0.133	0.160	0.075	0.037	0.003	9.990	9.994	0.011
PBS	0.202	0.132	0.111	0.053	0.020	0.008	0.009	9.990
	0.209	0.137	0.116	0.060	0.025	0.015	0.019	9.998
PBS	0.187	0.150	0.110	0.061	0.026	0.012	9.988	9.997
	0.190	0.154	0.112	0.064	0.037	0.013	9.997	0.011
1% FCS	0.544	0.135	0.080	0.065	0.015	9.995	9.998	9.998
+ PBS	0.572	0.159	0.104	0.092	0.040	0.022	9.985	9.989
1% FCS	0.232	0.126	0.092	0.071	0.020	0.012	0.005	9.988
+ PBS	0.225	0.116	0.082	0.062	0.009	0.005	9.994	0.001
1% FCS	0.206	0.162	0.112	0.050	0.007	9.985	0.002	0.002
+ PBS	0.227	0.184	0.134	0.070	0.030	0.007	9.980	9.990

PBS = Phosphate Buffered Saline

FCS = Fetal Calf Serum

Chapter IV

DISCUSSION

Our failure to produce a successful HRP conjugate may be attributed to any one of a number of lab-related factors such as errors in buffer preparation, temperature, pH or timing; however, these factors were carefully controlled and the procedures were tested more than once. The reagents employed were of the best quality and were well preserved when not in use. Uncontrollable inactivation of the biological functions of both enzyme and immunoglobulin may have occurred during manipulation of solutions or purification by gel chromatography; however, the number of researchers using the same techniques to obtain workable conjugates belies this possibility. The extremely low level of detectable protein (by the Biuret method [117]) contained in these conjugates and the evidence of poor coupling depicted by R.Z. values lower than those accepted by Nakane and Kawaoi [3] raise doubts as to the efficiency of the periodate conjugation method attempted.

A plausible explanation for our failures was revealed through the kinetic studies of Nadezhdin and Dunford [128]. The variability of enzyme preparations from batch to batch is in itself difficult to contend with when trying to standardize a procedure to obtain reliable and reproducible conjugates. In the normal enzymatic cycle (see Fig. 1), free energy favors the reduction process in which HRP-I is converted to HRP-II. Nadezhdin and Dunford [128] performed a series of experiments utilizing 0.01-0.05 M sodium periodate to convert HRP-II to HRP-I over a range of pH's. They also stated that appreciable denaturation of the enzyme occurs at periodate concentrations of greater than 0.1 M. Their results are at variance with Nakane's statement that HRP-II is desired for conjugation as the carbohydrate moiety involved in the aldehyde formation is much larger than that of HRP-I which is virtually nonexistent [129]. If this is the case, the presence of periodate converts HRP-II to HRP-I, thereby reducing the amount of enzyme with the necessary structure for conjugation.

Hevey et al. [94] and Sparacio et al. [130] have developed periodate conjugates for the detection of human α-fetoprotein with less success than Nakane and Kawaoi [3] in regard to losses of enzymatic and immunological activity. They report enzymatic activities of 14-18% and immunological activities of 10-18% being retained while Nakane and Kawaoi [3] report insignificant losses in both activities.

Regardless of the success others have achieved using HRP as a label for EIA, we found the enzyme to be unpredictable to work with for periodate conjugation. As a consequence of the above difficulties, the decision was made to investigate the parameters of the one-step glutaraldehyde conjugation of alkaline phosphatase which offers some advantages over horseradish peroxidase.

Horseradish peroxidase had been the enzyme of choice for labelling IgG molecules largely because of its relatively small size (40,000 daltons) which allowed penetration of cellular structures in histochemical preparations. Problems had arisen when using peroxidase conjugates because of the instability and deterioration of the enzyme in the presence of bacteriostatic agents [131]. Guesdon and Avrameas [73] reported wide fluctuations in reactions from test to test when using a peroxidase conjugate in identical test conditions and found alkaline phosphatase conjugates gave a great variation in enzymatic activity when a little variation in antibody occurred. With the advent of the ELISA in microtitre plates, alkaline phosphatase has been utilized since the inherent problems in achieving the ALP reaction in cell culture preparations are no longer significant [132]. The shelf-life of ALP conjugates tends to be longer than that of HRP conjugates because of its stability in the presence of bacteriostatic agents.

The ALP conjugates produced by the one-step glutaraldehyde procedures of Voller and collaborators [20] and Engvall and Perlmann [129] were remarkably similar. Although the E/P ratios varied slightly between the two methods and the amounts of the two proteins employed were different (1.5 mg ALP/0.5 mg IgG versus 5 mg ALP/2 mg IgG), the resultant conjugates were almost identical in enzymatic activity and molecular weight as evidenced by gel filtration through Sephacryl S-300. The advantage to using the method of Voller et al. [20] was the larger amount of conjugate obtained. Both methods employed a final glutaraldehyde concentration of 0.2% which was previously determined to be the optimal dilution for conjugation [38,98]. The quality of the glutaraldehyde used is also of consequence. A 25% solution available from Sigma comes in large volumes in screw cap bottles, hence oxidation of the linking agent becomes of significance through continued use. The ideal preparation is a 70% solution from Ingram-Bell which comes in small aliquots in sealed ampoules. We found use of the more purified glutaraldehyde reduces the amount of wastage of unused reagent. Whether the 25% or 70% glutaraldehyde is used, it is mandatory to dilute it to a 4.2% concentration before adding to the enzyme/IgG mixture in order to avoid unequal linking of the two protein molecules. Directly adding highly concentrated glutaraldehyde to the protein solution results in excessive linkage in the part of the solution to which the linking agent was first added and a lesser degree of linkage in the remaining solution as it diffuses through the mixture.

Gel filtration of the ALP conjugates through Sephacryl S-300 did separate the conjugate from other molecular species which were evident in the "trailing" effect seen in the separation peak. The presence of only a single peak lead us to believe that virtually all the ALP and IgG in the preparation were linked and although purification through gel filtration is not absolutely necessary, it is recommended in order to avoid any interference from unconjugated enzyme or immunoglobulin.

Voller and workers [20] used 100 ng/mL of human IgG to coat polysterene microtitre plates (Dynatech) for the determination of the working strength of the conjugate. Our conjugate titrations consistently showed no binding occurring at IgG concentrations of less than 1 μg/mL and our endpoint dilutions were determined using a 10 μg/mL coating of IgG in accordance with the work of Luzzio and collaborators [19] who employ 10 μg/mL of leishmanial antigen for endpoint determination of conjugates. Yolken and collaborators [133] and Keren [134] were also unable to produce a conjugate sensitive enough to employ 100 ng/mL of IgG and reported IgG concentrations between 1 μg/mL and 10 μg/mL for working strength determinations.

In addition to the reported conjugates (Tables 3, 4 and 5), several other conjugates were prepared, their enzymatic activities determined and their endpoint titres were determined. The enzymatic activity of the conjugates ranged from 195-510 IU/mL and the difference in activities was attributed either to variations in the enzyme preparations or to the use of plastic micro-centrifuge tubes which may have bound some of the enzyme molecules and reduced the amount of ALP available for reaction. In direct relation to the enzymatic activities, the corresponding conjugate endpoint dilutions ranged from 1:100 to 1:500. Retention of original enzymatic activity also varied from 16-20% in conjugates with lower activity to 65% in those conjugates having an enzymatic activity of 510 IU/mL. The amount of specific enzymatic activity was directly related to the endpoint conjugate dilution, yet none of the papers reviewed gave any indication of what the enzymatic activities of the conjugates employed were. Only the conjugate endpoint dilutions were revealed and these ranged from 1:20 [67] to as high as 1:3000 [135]. Granfors [107] used his swine IgG conjugate at 1:200 which is in sharp contrast with Leiniki and Pässilä [102] who used theirs at a 1:2800 dilution. The working strength of Yolken and workers' goat anti-GP IgG-ALP conjugate was 1:400 which correlates closely with the endpoint dilution of 1:500 we used for our goat anti-GP IgG conjugate. Since the enzymatic activity was a measure of the biological function of ALP, we felt that such a determination should be made for each conjugate preparation as an indication of the biological strength of the conjugate and the endpoint titre should reflect the "measure" of strength.

At the beginning of our investigation, we had difficulty in establishing the criteria for the "negative cut-off" value of our controls to be used in the determination of the endpoint titre of antigens and antisera assessed by checkerboard titration. The difficulty lies in the great variation of criteria employed by different authors in establishing the endpoints of their systems. Particularly puzzling were the results reported by Forghani and collaborators [77, 106]. These investigators defined the endpoint antibody titre of their antisera by visual and by spectrophotometric readings. With the visual reading, the endpoint antibody titre consisted in the highest serum dilution showing visible color and the reaction was considered specific only if the corresponding wells containing uninfected control cell antigen showed no color at all. When the spectrophotometric reading was employed, the endpoint antibody titre was the dilution which had an absorbance value ≥0.060 provided that the corresponding uninfected control cell antigen had an absorbance of ≤0.030. In other words, if the absorbance of the test was two or more times greater than the absorbance of the corresponding control, the result was considered to be positive with no regard to a pre-established "cut-off" negative value. After repeated testing of our antigens and antisera we found Forghani's criteria unacceptable for our purposes. Yolken et al. [42] reported a positive/negative value (P/N) determined by dividing the absorbance of the test by the absorbance of a known negative sample. If the P/N value was ≥2.0, the test was considered positive. Likewise visual reading was positive if the color of the test was a darker yellow than that of the negative control. No absolute negative "cut-off" absorbance values were ever reported for the control systems; therefore it was impossible to ascertain the true meaning of their positive values not having any negative upper limit absorbance on which to base a comparison. The same vague evaluation of positive findings was reported by Kraaijveld et al. [136] who also used a two-fold or greater difference between the absorbance of test and negative control wells as a measure of positivity. Parker and workers [63], on the other hand, established a more definitive approach in defining a positive reading which consisted in an absorbance value of equal or greater than 0.150 after the absorbance of the negative control well was subtracted from the absorbance of the test well. Our findings in repeated antigen/antisera titrations were consistent with the values established by Parker and workers [63]. Like Parker, therefore, we accepted as a negative "cut-off" endpoint an absorbance value of 0.150. Conversely, a positive value was any absorbance value greater than 0.150 provided the absorbance was at least twice as much as the one of the negative control.

A complete set of control checks on all reagents and procedures used for the ELISA test was of utmost importance when performing the antigen/antisera checkerboard titrations. The antigen controls ensured that the conjugate did not bind non-specifically to HeLa cell components in the absence of immune serum and that the cellular material itself did not absorb at 405 nm. Likewise, the normal and immune sera controls indicated that these elements did not bind to the plastic even when high concentrations of both were employed. Normal serum added to infected and normal HeLa cells ensured that the GP anti-Coxsackie B-viruses antisera were binding to the Group B Coxsackieviruses and not just to the HeLa cells. All the above controls had absorbance values of zero (Tables 7-12) and confirmed the immunological specificity of both the antisera and the conjugate. The use of normal HeLa cells in the test was mandatory for two reasons, the first being as a negative control to which the test results were compared for evaluation of positivity and, secondly, as a monitor for non-specific adsorbance of the immune sera since the sera were produced using antigen prepared in HeLa cells and could conceivably have contained anti-HeLa cell immunoglobulins. The results of the antigen titrations ensure that the reaction of the antisera is specific. The use of controls by other authors appears to be sadly lacking or is never mentioned. We seriously doubted that a complete set of controls was included on each plate, rather that a set of controls was run on a separate plate and the results used for however many test plates were included in the run.

The optimal antigen concentrations for Coxsackieviruses B1, B2 and B4-B6 were at a 10^{-3} dilution of the antigens prepared in HeLa cell culture, whereas for the Coxsackievirus B3, a 10^{-2} dilution was employed. Dilutions of 10^{-2} (except for Coxsackievirus B3) gave non-specific background reactions in the normal HeLa cell controls and the antigen/antibody concentrations were not optimal until higher dilutions of the antigen were employed. On the other hand, a dilution of 10^{-4} did not guarantee sufficient antigen to be detected. Frazier and Shope [137] indicate that arbovirus antigens are not adequate for use in the ELISA unless they have a titre of 10^{6} PFU's. All the antigens used in this study had titres of at least 10^{6} $TCID_{50}$ and it is likely that a lower titre would have resulted in antigen titrations where the antigen could not be diluted enough to eliminate non-specific interaction without also diluting out the positivity of the results. The endpoints for optimal antigen concentration determined for each Coxsackie B-virus were very clear-cut and indicated that the test may have an important applicability for the detection of antibody to Coxsackie B-viruses.

Without having used a new lot of polysterene plates when attempting to test Group B Coxsackieviruses prepared in AGMK cells, we might never have run into the problem of non-specificity or recognized how random and erratic an occurrence the problem was. The problem of non-specificity in serological tests has long hindered many workers; the ELISA is not exempt but in our investigation the non-specificity stems from the plastic itself rather than factors inherent in the reagents. The fact that the first set of antigen titrations resulted in clear endpoint dilutions with background interference only at the highest concentrations of antigen and antiserum and the second attempt resulted in false positive reactions in all wells containing sera (either normal or immune) sharply indicates the inconsistency of the polysterene plates employed. Recently, the problem of inconsistent plate coating has become more publicized and various workers are encountering the same difficulties. Chessum and Denmark [138] tested several batches of polysterene plates and have found that antigen unexplicably binds unevenly to them. Each of the batches was identically tested with the same reagent preparations and dilutions and reasonably well-correlated results were expected. The plates were divided into three different groups on the basis of the results. The first group demonstrated an "edge" effect wherein the edge wells of the plate show unusually high absorbance readings not attributable to dessication or temperature variation. The second group shows high readings in the middle, bottom of the plate with low readings at the top and sides. The last batch showed only a couple of randomly placed high absorbance values and was considered reliable for use in antibody quantitation. The situation is further compromised as Murphy et al. [139] reported that 5-10% of plates *from the same lot* bound test reagents non-specifically and gave high background readings. Almeida et al. [140] also warns against variations from batch to batch of all types of plastic microtitre plates. They recommend that a sample of each batch be tested using a reference serum IgG, the conjugate and the substrate. A standard deviation of 0.05 with an absorbance reading of 1.0 may be considered acceptable. Assurance of obtaining reproducible results may be attained through the use of covalent linking techniques rather than passive adsorption.

The duplicate readings obtained from the Flow Titretek Multiscan photometer often varied considerably, by as much as 0.100 units of absorbancy. We now realize that these differences arose from the unequal binding properties of the plastic, although the conclusions drawn from the readings were valid since definite trends in absorbancy were easily recognizable and replicate tests confirmed the findings.

Brandt et al. report that 73% of their positive ELISA results for rotavirus detection were false positives and the option for improving the specificity of the test was by employing a confirmatory test involving a "capture antibody" [141]. Use of covalent linking techniques rather than passive adsorption for coating of the plastics ensures reproducible results according to Almeida et al. [140] and Lehtonen and Viljanen [22]; however, in our opinion, ELISA using passive adsorption can be very effective provided the problems are completely understood and the appropriate precautions are taken to avoid misinterpretation of the test results. For future work, the outside wells of each plate will not be used since this area of the plates seems to be particularly subject to inconstant protein binding.

Many researchers add 1% fetal calf serum or bovine albumin to their buffers for diluting the antisera to be used in the ELISA test [66,106,142] in order to minimize the degree of non-specificity encountered. The addition of 1% FCS to our diluent did nothing by way of reducing non-specific adsorption of the GP serum (Tables 15-17). The fact that the normal serum bound to the degree it did even at a pH of 7.2 when theoretically a pH of 9.6 is optimal, indicates that there is a strong interfering adsorbance factor inherent in the plastic. For our purposes, addition of FCS to the antisera diluent had no advantage over adding only a non-ionic detergent as Voller et al. did in their protocol [143]. Herrman and Collins [100] warned against using excessive amounts of inhibitor protein since some serum proteins may interfere with the immunoassay proper; therefore, the lowest effective amount of inhibitor protein should be used. Post-treatment of the coated plates with 5% bovine albumin helped minimize non-specific reactions by blocking the remaining sites on the plastic after the coating protein was already adsorbed. This procedure eliminated the need for adding inhibitor protein to the diluents where they might have directly interfered with the antigen/antibody binding process. One percent bovine albumin was added to the conjugate diluent for stability since the incubation period was only 30 min long and we felt any deleterious effects would be insignificant. The results of the conjugate titrations as well as the antigen titrations supported this reasoning.

Reports of non-specific binding are not limited to non-commercial laboratories but extended also from those employing commercial conjugate preparations [144]. The reliability of each kit was different from the next and, in some cases, was not as reliable as non-commercially-prepared conjugates. The stress, though, was on the lack of improvement in the solid phases available for ELISA tests and the problem of inconstant selective adsorption of antigens and antibodies remains unsolved. Even with the use of commercial preparations, there is a clear need for standardization of materials and methods in order to bypass the confusion arising from a host of procedures used by numerous workers.

Although lack of time and the erratic results obtained because of non-specificity of the plastics prevented cross-reactivity testing of the Coxsackie B-viruses, the preliminary background for ongoing work to use the ELISA technique for antigen detection in animal tissue, tissue culture and eventually for human antibody detection has been defined. The type and extent of the problems involved have been fully realized and, at the same time, the applicability of the test to our purposes has been proven. Future work involves the development of a technique to control the non-specificity of the plastics in such a way as to provide consistent, reproducible results.

CONCLUSIONS

The periodate method of conjugation using horseradish peroxidase was unsatisfactory for our purposes as it yielded conjugates with low R.Z. values and low protein content. In our study, usable conjugates were obtained by the one-step glutaraldehyde conjugation procedure and alkaline phosphatase. The enzymatic activity of these conjugates correlates directly to the conjugate titre and both parameters should be determined to evaluate the degree of retention of enzymatic biological function. Variations in enzymatic activity from conjugate to conjugate were thought to depend on the characteristics of the alkaline phosphatase preparations. Passage of the conjugate through Sephacryl S-300 superfine columns did help remove unconjugated ALP and IgG as well as other factors which may have interfered with the ELISA reaction.

The key to successful performance of the ELISA test lies in the strict monitoring and control of the non-specificity of reactions whether it be due to the immune reagents, buffered diluents, antigens or the solid phase employed. The control of all but the last variable can be achieved by including a complete set of controls on *each* plate used for the ELISA test. The controls include: antigen (infected and uninfected culture cells), normal and immune sera and normal serum-antigen. Non-specific binding of proteins to plastic solid phases cannot be overcome by addition of bovine albumin to buffer diluents. Two solutions to the problem are currently available:

1. the use of covalent binding techniques to coat the solid phase.
2. the testing of each lot of plates used as described [140].

In our opinion, the passive adsorption technique of protein coating and testing of the plate batches is an appealing alternative to covalent binding techniques. Refraining from using the outside row of wells on each plate may help to eliminate some non-specific reactions.

Absorbance values for determining positivity and negativity of reaction should be absolute to enable comparison and consistency of test results. For our purposes, a positive result was defined as being two times the absorbance of the negative controls provided the absorbance of the latter was not greater than 0.150 units.

The endpoints for optimal antigen and antiserum concentrations for each Coxsackie B-virus were clear-cut and indicated that the ELISA test may have an important applicability for the detection of Coxsackie B-viruses in culture cells as well as in the assessment of acute and late infection in human sera.

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APPENDIX

A. PROTEIN A-SEPHAROSE

1. Phosphate Buffered Saline, pH 7.4 (0.01 M PO_4, 0.13 M NaCl)

Soln A	1.0 M KH_2PO_4	136.08 g/L
Soln B	1.0 M K_2HPO_4	174.18 g/L

Mix 1.68 mL of Soln A, 9.2 mL of Soln B and 7.6 g of NaCl. Bring up to one litre using deionized water. Add 5×10^{-5} M Thimerosal.

2. Glycine-HCl, pH 2.8 (0.01 M)

Soln A	2.0 M glycine	150.14 g/L
Soln B	1.0 M HCl	

Mix 50 mL of Soln A with 33.6 mL of Soln B. Bring up to one litre with deionized water.

3. Carbonate-Bicarbonate, pH 9.3

$NaHCO_3$	17.3 g
Na_2CO_3	8.6 g

Dissolve in one litre deionized water.

B. PERIODATE CONJUGATION

1. Sodium bicarbonate, pH 8.1 (0.3 M)

126 mg $NaHCO_3$ dissolved in 5 mL deionized water.

2. Sodium periodate (0.06 M)

64 mg $NaIO_4$ dissolved in 5 mL deionized water.

3. Phosphate Buffered Saline, pH 7.2 (0.005 M PO_4, 0.15 M NaCl)

NaCl	8.5 g
Na_2HPO_4 (anhydrous)	0.565 g (~ 0.004 M)
KH_2PO_4	0.135 g (~ 0.001 M)

Dissolve the NaCl in 50 mL deionized water, add the Na_2HPO_4 and KH_2PO_4 to make one litre with water and record the pH. The pH may vary from 7.1 - 7.5.

C. ELISA

1. Carbonate-bicarbonate, pH 9.6 (0.07 M CO_3)

This is the buffer used to dilute protein for coating the plates.

Soln A	1.0 M $NaHCO_3$	84.0 g/L
Soln B	1.0 M NaH_2CO_3 (anhydrous)	106.0 g/L

Mix 45.3 mL of Soln A with 18.2 mL of Soln B and bring up to one litre with deionized water. Check the pH.

2. Substrate - OPDA

A 1% solution of orthophenylene diamine (OPDA) was made in methanol. A 1:1000 dilution of the OPDA solution was made in deionized water. Dilute 30% H_2O_2 to 5% with deionized water. Add 0.1 mL of the 5% H_2O_2 to 100 mL of the diluted OPDA to complete the substrate.

3. Tris-HCl, pH 8.0 (0.05 M)

Soln A	0.2 M Tris	24.23 g/L
Soln B	1.0 M HCl	

To 240 mL of Soln A add 26.8 mL of Soln B, bring up to approximately 900 mL with deionized H_2O and put at 4°C to cool. Once cooled, pH to 8.0 using 1.0 M HCl and q.s. to one litre.

4. AMP, pH 10.3 (0.782 M)

2-amino-2-methyl-1-propanol	34.838 g
1 M HCl	100 mL

Add HCl to AMP and bring volume up to almost 500 mL, check pH and adjust to 10.3 using 1 M HCl or 1 M NaOH. Q.s. to 500 mL with deionized H_2O.

5. Substrates - pNPP

a. 1 mg/mL - one Sigma, #104 pNPP tablet was added for every 5 mL of assay medium.

b. 3.71 mg/mL - 0.1855 g of Sigma, #104-0 pNPP was added to 50 mL assay medium.

c. assay medium - 0.075 mL of 1 M $MgCl_2$ were added to every 50 mL of 0.782 M AMP buffer for a final concentration of 1.5 mM $MgCl_2$.